The feeding value for dairy cows and the agronomic performance of white clover (*Trifolium repens* L.) selected for increased floral condensed tannin

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VICTORIA G. BURGGRAAF

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Abstract

ABSTRACT

Legumes containing 20 to 40 g of condensed tannin (CT) per kg of dry matter (DM) can improve dairy cow milk production by reducing ruminal protein degradation to ammonia and preventing bloat. White clover (*Trifolium repens* L.) produces CT in its flower heads. High tannin (HT) white clover, bred for increased flowering and increased floral CT concentration, was evaluated under dairy grazing in Hamilton, New Zealand. Its performance in monoculture was compared to that of Grasslands Huia white clover over two years, and five short-term grazing experiments determined its effects on Friesian dairy cows.

Huia and HT had similar floral CT concentrations, ranging from 15 to 77 g/kg DM over two flowering seasons. HT clover had higher flower densities than Huia until the second summer after sowing, resulting in higher clover (leaf plus flower) CT concentrations. Clover CT peaked at 12.1 g/kg DM for HT and 5.7 g/kg DM for Huia. HT swards had lower stolon growing point densities than Huia swards and annual DM yields averaged 10.0 and 11.0 t DM/ha for the respective clovers. The ingress of non-sown white clover genotypes reduced treatment differences in the last 10 months of the experiment.

Mild bloat occurred in cows grazing both clovers. Cows grazing HT white clover had rumen ammonia concentrations 5 to 26% lower than that of cows grazing Huia, indicating less proteolysis in the rumen of HT cows, but there were no consistent effects on rumen soluble protein or volatile fatty acids (VFA). Differences between treatments in dietary CT concentrations were too small to affect milk production or composition.

Minced mixtures of 0, 25, 50, 75 or 100% of DM as white clover flower with the remainder as white clover leaf, were incubated *in vitro* and rumen metabolite concentrations determined at 0, 2, 4, 8, 12 and 24 hours. Polyethylene glycol was added to one of the 50% flower treatments to inactivate CT. Clover flowers had less soluble protein than leaves at 0 hours, and increasing the percentage of flowers from 0 to 100%

reduced the net conversion of plant-N to ammonia-N from 29 to 12%. The contribution of CT to these effects was small. Increasing percentages of clover flowers did not significantly affect total VFA production but increased acetate to propionate (A:P) ratios. White clover CT decreased A:P ratios. In another *in vitro* experiment perennial ryegrass leaf (*Lolium perenne* L.) was incubated either alone or with white clover flowers or birdsfoot trefoil (*Lotus corniculatus* L.). Clover flowers were more effective at reducing proteolysis than birdsfoot trefoil, due largely to less release of soluble protein, but birdsfoot trefoil treatments had the lowest A:P ratios.

In conclusion, HT clover had higher forage CT concentrations than Huia because of increased flowering. Increased flowering reduced the agronomic performance of HT and lowered rumen ammonia concentrations, but did not increase milk production or prevent bloat. White clover flowers reduced rumen proteolysis *in vitro*, but this was mainly a result of their low protein concentration. White clover CT and birdsfoot trefoil forage benefited the molar percentages of VFA, but increasing the proportion of clover flowers did not. Further increases in white clover CT concentrations may benefit ruminant performance, but this should not be implemented through increased flowering.

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LIST OF ABBREVIATIONS

A	acetate
ADF	acid detergent fibre
a.i.	active ingredient
A+B:P	(acetate plus butyrate) to propionate ratio
A:P	acetate to propionate ratio
Apr 02	April 2002 animal experimental period
Apr 03	April 2003 animal experimental period
B	butyrate
BT	birdsfoot trefoil
°C	degrees celcius
C_{31}	hentriacontane alkane
C_{32}	dotriacontane alkane
C_{33}	tritiacontane alkane
Ca	calcium
CHO	soluble carbohydrate
cm	centimetre (10^{-2} m)
CO_2	carbon dioxide
CP	crude protein
CT	condensed tannin
Dec 01	December 2001 animal experimental period
Dec 02	December 2002 animal experimental period
dL	decilitre (10^{-1} L)
DM	dry matter
F	white clover flower
Feb 02	February 2002 animal experimental period
g	gram
x g	gravitational field
H	hydrogen
h	hour

ha	hectare (10 000 m^2)
HCl	hydrochloric acid
H_2O	water
HT	high floral tannin white clover
Huia	Grasslands Huia white clover
K	potassium
kg	kilogram (10^3 g)
kJ	kilojoule
L	litre
LSD	least significant difference
m	metre
ME	metabolisable energy (MJ/kg DM)
Mg	magnesium
mg	milligram (10^{-3} g)
MJ	megajoule
mL	millilitre (10^{-3} L)
mm	millimetre (10^{-3} m)
mM	millimole (10^{-3} M)
MUN	milk urea nitrogen
N	nitrogen
NAN	non-ammonia nitrogen
NDF	neutral detergent fibre
NH_3	ammonia
NIRS	near infrared spectroscopy
OH	hydroxide
OMD	organic matter digestibility
P	probability statistic
P	phosphorus (when used in Section 3.3.2)
P	propionate (when reporting on volatile fatty acids)
PEG	polyethylene glycol
R	perennial ryegrass leaf
rpm	revolutions per minute
S	sulphur
SED	standard error of the difference

t	tonne
µL	microlitre (10^{-6} L)
µm	micrometre (10^{-6} m)
VFA	volatile fatty acid
VFI	voluntary feed intake

LIST OF PLANT SPECIES CITED

Common name	Scientific name
Annual poa	*Poa annua* L.
Birdsfoot trefoil	*Lotus corniculatus* L.
Broad-leaved dock	*Rumex obtusifolius* L.
Carob	*Ceratonia silique* L.
Chickweed	*Stellaria media* L.
Chicory	*Cichorium intybus* L.
Cicer milkvetch	*Astragalus cicer* L.
Dandelion	*Taraxacum officinale* Weber.
Erect dorycnium	*Dorycnium rectum* L.
Hybrid ryegrass	*Lolium* x *hybridum*
Lentil	*Lens culinaris* Medik.
Lotus major	*Lotus pedunculatus* Cav.
Lucerne, alfalfa	*Medicago sativa* L.
Maize, corn	*Zea mays* L.
Mimosa	*Acacia spp*
Mulga	*Acacia aneura* Benth.
Perennial lupin, lupin	*Lupinus polyphyllus* L.
Perennial ryegrass, ryegrass	*Lolium perenne* L.
Quebracho	*Aspidosperma quebracho* Schlecht.
Red clover	*Trifolium pratense* L.
Redroot	*Amaranthus retroflexus* L.
Sainfoin	*Onobrychis viciifolia* Scop.
Sheep's burnet	*Sanguisorba minor* Scop. ssp. *Muricata*
Sow thistle	*Sonchus olearaceous* L.
Sulla	*Hedysarum coronarium* L.
Summergrass	*Digitaria sanguinalis* L.
Tamarind	*Tamarindus indica* L.
Tobacco	*Nicotiana tabacum* L.
White clover, clover	*Trifolium repens* L.
Yorkshire fog	*Holcus lanatus* L.

CHAPTER 1

General introduction, objectives and thesis format

CHAPTER 1: General introduction, objectives and thesis format

1.1 GENERAL INTRODUCTION

White clover (*Trifolium repens* L.) is a high quality legume, generating high levels of animal production when fed as a sole diet or with grasses (Ulyatt et al. 1977). However, white clover has a propensity to cause bloat, and much of the plant protein is degraded to ammonia in the rumen then excreted as urea in urine (MacRae & Ulyatt 1974). High urinary nitrogen is detrimental to the environment and there is an energetic cost to the animal of urea synthesis that could otherwise be used for production. In New Zealand, ruminant production is based mainly on mixed pastures of white clover and perennial ryegrass (*Lolium perenne* L.). In spring and autumn protein concentration and digestibility of pasture is high, leading to much of the protein ingested being degraded and the nitrogen excreted. In summer, low protein and high fibre concentrations in pasture may limit milk production.

Feeding forages containing condensed tannins (CT) can prevent bloat, and the ability of CT to bind with proteins reduces plant protein degradation by microbes in the rumen. The increased flow of amino acids to the intestines for absorption often improves animal performance (Waghorn et al. 1999). The legume birdsfoot trefoil (*Lotus corniculatus* L.) contains CT, and when fed to dairy cows, milk production is higher than from cows fed perennial ryegrass or white clover (Harris et al. 1998), partly because of the CT (Woodward et al. 1999; 2000). In some cases, CT may increase milk protein and reduce milk fat concentration (Woodward et al. 2000). Although these attributes are valuable to the dairy industry, the use of birdsfoot trefoil and other forage legumes containing CT is not common, as they are difficult to establish and manage, and do not persist in competition with ryegrass, white clover and weeds in fertile soils.

CT differ between plant species in their concentration and structure, exerting different effects on the animals that graze them. Positive effects on ruminant health and

productivity generally occur with concentrations of 20 to 40g/kg DM (Aerts et al. 1999). White clover produces CT in its flowers, but forage concentrations are low compared with other legumes that contain CT. Also, flower production is seasonal, and flowers are normally only a small proportion of clover dry matter.

High floral tannin (HT) white clover was recently bred to increase the concentration of CT in white clover forage, by increased flowering intensity and duration and increased flower head CT concentrations. HT white clover may be a viable option for increasing the CT concentration of temperate pastures, as farmers are already familiar with the management of this species. However, achieving increased flowering and CT production in white clover may limit its agronomic performance. White clover persists by producing horizontally spreading stolons. Stolons are formed in the leaf axils, which are also the site of flower production, so for each flower produced a site for stolon branching is lost.

Before HT clover can be released onto the commercial market, thorough testing is required. New forage cultivars must be evaluated under the conditions that they must perform. Evaluation of new white clover cultivars in the past has focussed mainly on their agronomic performance. When breeding for feed quality components such as CT, evaluation of animal performance is essential. This thesis evaluates the agronomic performance of HT clover, and the performance of dairy cows grazing this clover. Agronomic performance can vary within and across years, so data were collected over two years. The response of dairy cows also varies over the milking season, and with changes in feed quality, so animal experiments were repeated within and between years. Clover flowers differ in nutritive value to clover leaves, so varying proportions of clover flowers in the diet may affect animal performance. *In vitro* experiments were used to determine the specific effects of white clover flowers and white clover CT on forage digestion in the rumen, and these were compared with the effects of birdsfoot trefoil and its CT when mixed with perennial ryegrass.

1.2 OBJECTIVES

The agronomic performance of HT white clover and its effects on grazing dairy cows were compared to that of a control cultivar (Grasslands Huia white clover). Assessments were made in monoculture to avoid complications due to varying percentages of clover in different treatments. Initial results from the grazing experiments raised some questions that were addressed by *in vitro* experiments. The specific objectives of this research were to:

1) Determine the within and between year variation in flowering and condensed tannin concentration of HT white clover compared to that of Huia.
2) Evaluate the morphology, growth and persistence of HT white clover compared to Huia, with emphasis on dry matter production, stolon density and the effects of increased flower production on stolon branching.
3) Determine whether or not HT white clover prevents bloat and reduces protein degradation in the rumen, and evaluate its effects on dairy cow milk production and composition.
4) Determine the effects of increasing the proportion of white clover flowers in a white clover diet on rumen digestion, and evaluate the contribution of condensed tannins to these effects.
5) Compare white clover flowers to birdsfoot trefoil forage as a means of reducing excessive rumen proteolysis, and determine the effects on the production of energy yielding substrates when included in mixtures with perennial ryegrass.

1.3 FORMAT OF THE THESIS

The thesis consists of seven chapters. Following this introduction, Chapter 2 presents a review of literature reporting information on the growth and morphology of white clover and its nutritional benefits and problems as a feed source for dairy cows. Condensed tannins are reviewed as a solution to the problems of bloat and excessive ruminal white clover protein degradation. The effects of CT on feed digestion and animal performance and methods of incorporating CT into New Zealand agriculture, with emphasis on increasing CT concentration and flowering in white clover are

discussed. Finally, the breeding programme for HT white clover and methodologies for evaluating new cultivars containing CT are reported.

Chapter 3 assesses the agronomic performance of HT white clover, compared to Huia. Evaluations are made of dry matter production, flowering, persistence, morphology and condensed tannin concentrations of the plants over two years. Details of the experimental site and pasture management are also included in this chapter.

Chapter 4 evaluates HT white clover as a feed source for grazing lactating dairy cows. Its effects on bloat, feed intake, rumen digestion, and milk production and composition are assessed in 5 short-term grazing experiments over two years, using Huia white clover as a control.

The diets fed in each experiment in Chapter 4 varied in CT concentration and the proportion of clover flowers. Chapter 5 reports on the effects of increasing percentages of white clover flowers in clover herbage on rumen soluble protein and ammonia concentrations and the production of energy yielding substrates (volatile fatty acids; VFA) for the animal. Assessments were made *in vitro* and the effects of white clover floral CT were quantified.

White clover is normally fed in mixed pastures with perennial ryegrass. Excessive protein degradation may also occur from perennial ryegrass leaves, so Chapter 6 assesses the effects of mixtures of white clover flowers with perennial ryegrass leaves on protein degradation and VFA production in comparison to a mixture of birdsfoot trefoil and perennial ryegrass. The effects of the CT in birdsfoot trefoil on these processes were also evaluated.

The final chapter (Chapter 7) integrates and summarises all experimental material. Publications and other relevant material from this thesis are presented in the appendices, followed by a bibliography of all information referenced in this thesis.

CHAPTER 2

Literature review

CHAPTER 2: Literature Review

2.1 INTRODUCTION

To remain internationally competitive, the New Zealand dairy industry has to grow large amounts of quality pasture that can be grazed throughout the year (Caradus & Clark 2001). Mixtures of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) dominate these pastures. White clover contributes to the low cost of production by fixing atmospheric nitrogen, reducing the requirement for applications of nitrogen fertiliser, and by providing high quality forage that results in higher milk yields than from grasses alone (Harris et al. 1997).

However, mixed perennial ryegrass/white clover pastures constrain dairy farm productivity in spring and autumn, when high concentrations of dietary protein can cause bloat, and much of this protein is degraded in the rumen and then excreted in urine at a metabolic cost to the animal. In contrast, in summer, protein supply to the animal may be lower than that required to sustain high levels of milk production (Moller et al. 1996).

This literature review provides a background to the growth, morphology and persistence of white clover, as well as its benefits to animal production, animal health and excessive protein degradation. Legumes containing condensed tannins (CT) protect protein from excessive microbial degradation in the rumen and can benefit animal performance. The benefits and agronomic limitations of plants expressing CT are reviewed.

White clover with increased CT concentrations is proposed as a means of increasing the nutritive value of pasture. Methods for increasing CT concentrations in white clover, in particular through increased flowering, are discussed. The breeding of the high floral tannin (HT) white clover used in this research is outlined, as well as procedures for evaluating plants containing CT.

2.2 WHITE CLOVER AS A FEED FOR DAIRY CATTLE

2.2.1 The origin of white clover and cultivar development

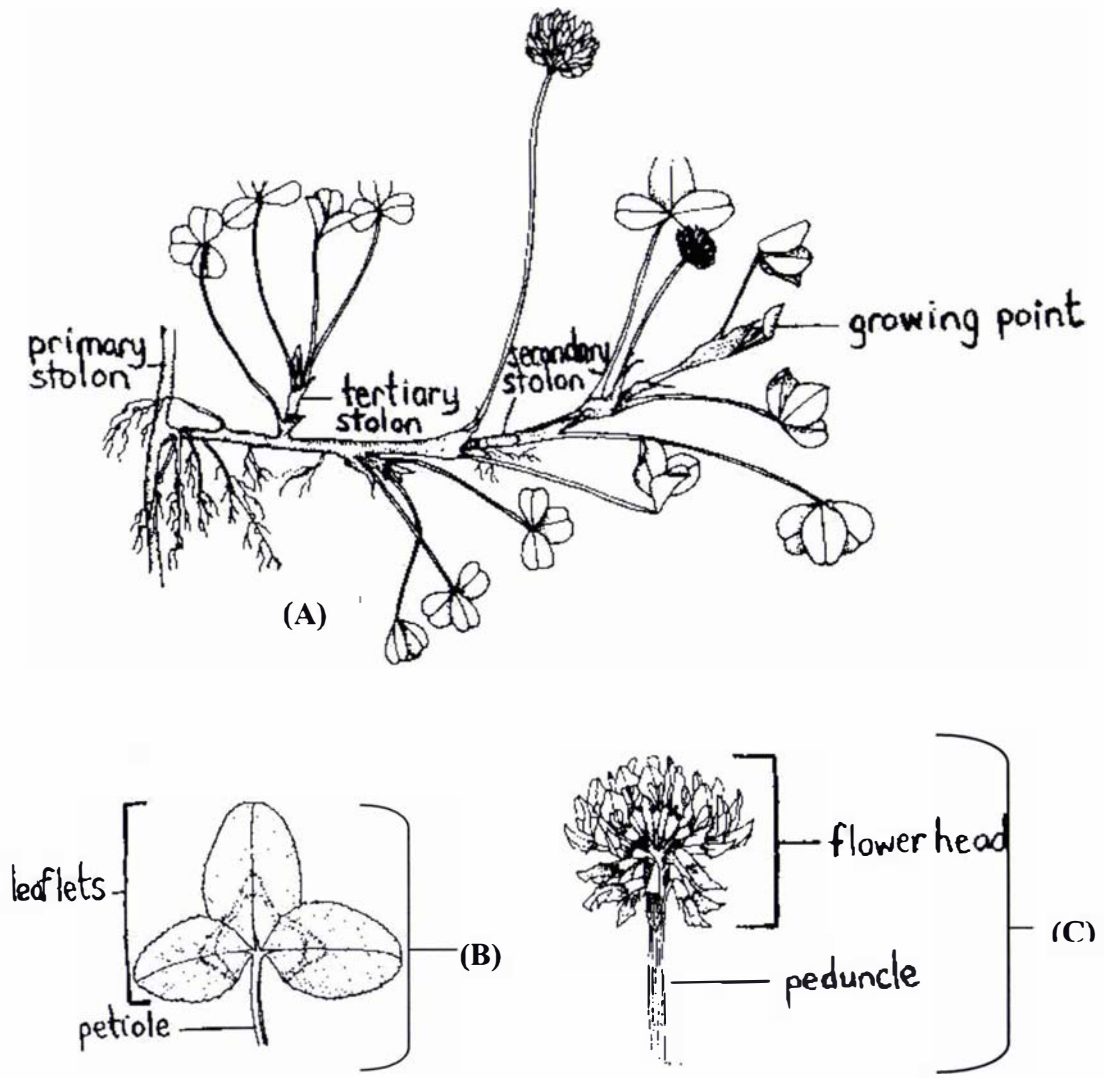
White clover is a temperate perennial legume, originating from the Mediterranean region (Vavilov 1951), and it has become widely adapted to regions as diverse as the Arctic and the subtropics (Williams 1987a). White clover was introduced to New Zealand from England in the mid 19th century, and has since become the most important pasture legume (Williams 1987a). White clover benefits pasture by fixing nitrogen and by providing complementary growth to grasses that is of a high nutritive value (Caradus et al. 1996).

The identification of white clover strains and ecotypes in New Zealand began in the 1920's. Since then, more than 250 synthetic cultivars and ecotypes of white clover have been released worldwide (Caradus 1986). Grasslands Huia, a medium-leaved cultivar with widespread adaptation, was released in 1957 and currently accounts for 70% of white clover seed exported from New Zealand (Pyke et al. 2004). New Zealand white clover breeding programmes have aimed at improving yield, competitive ability and persistence, with genetic gains made in these traits averaging 6% per decade (Woodfield & Caradus 1994).

2.2.2 White clover growth and morphology

The primary growth unit of white clover is a phytomer consisting of a node on a stolon, able to vegetatively reproduce. Stolons grow by elongation of internodes. At the apical meristem (growing point) new nodes are continuously developed. The nodes of a stolon each produce a single leaf, an axillary bud and two root primordia (Thomas 1980). Usually, one of these primordia develops into a root, and either a stolon branch or a flower can develop from the axillary bud (Figure 2.1). Each stolon branch can produce all of the same structures as the parent stolon, and thus can live as an independent unit if it becomes isolated from the rest of the plant (Thomas 1980).

FIGURE 2.1 Morphology of a white clover plant, showing (A) whole plant with primary, secondary and tertiary stolons, (B) leaf, (C) flower. Adapted from Langer (1990).



Seedlings develop to form a crown of stolon branches on top of a central tap root (Thomas 2003). White clover plants extend vegetatively into favourable niches by lateral extension and branching of stolons (Brock & Hay 2001). About 1 year after sowing, death of the tap root occurs in some plants, splitting the initial plant into several smaller clonal plants consisting of one primary stolon, with up to five levels of branching. Clonal plants continually grow from the growing point and decay at the stolon base. They rely on small nodal roots for anchorage and nutrient and water uptake and hence are more vulnerable to stress (Brock & Hay 2001).

During winter growth is slow, and a large proportion of stolon is buried (Hay et al. 1987). In spring up to 70% of stolon material senesces causing fragmentation of plants (Hay et al. 1989), and many of the resulting small plants die. This fragmentation period can be displaced or not occur at all, depending on weather conditions. Surviving plants then grow and branch rapidly over summer (Brock et al. 1988).

2.2.3 White clover persistence

In temperate climates regeneration of white clover is primarily by vegetative production of new stolons (Harper 1978). However, substantial establishment of white clover from seed has been observed after pasture damage from drought (Harris 1987). Densely branched cultivars are normally the most persistent, but they may have smaller leaves and lower forage yield potential (Rhodes & Harris 1979). The development of new stolon branches is controlled by light conditions experienced at the node from which they develop (Robin et al. 1994), with shading leading to fewer branches and less well developed stolons. In mixed swards the rapid growth of associated grasses in spring must be controlled with frequent grazing to prevent excessive shading of clover (Harris & Clark 1996).

Stolons are the primary storage organs of the clover plant, and exchange of carbohydrates occurs between stolons (Chapman et al. 1992). This provides the plant with a buffer against stress factors (Chapman et al. 1992), and the more profusely branched the plant, the greater the buffering capacity. Flower production may therefore reduce plant persistence, as for each flower produced a site for stolon branching is lost.

During summer white clover has a competitive advantage over most temperate grasses because of its higher optimum temperature for growth. However, this advantage is only realised under adequate soil moisture (Harris 1987). The shallow root system of white clover makes it susceptible to drought stress, especially in spring when plants are small (Brock 1988). High soil surface temperatures and soil moisture deficits can cause the collapse of stolon populations. High stolon growing point densities, large tap roots and high root to shoot dry matter ratios have been identified as traits that can improve white clover persistence under short-term moisture deficits (Brock 1988; Woodfield 1994).

White clover persists best on moderately-low to high fertility soils, with most soils requiring annual maintenance applications of phosphorus and sulphur to ensure adequate growth, with potassium required in intensively grazed systems such as dairying (Woodfield & Caradus 1996). Lime is normally required for white clover establishment, and once established optimum growth occurs at a soil pH of 5.5 to 5.8 (Woodfield & Caradus 1996). White clover vigour and persistence is also affected by nematodes, slugs, insect pests and viruses. The introduction of the clover root weevil (*Sitona lepidus*) to New Zealand in the 1990's has caused widespread damage to North Island white clover populations (Eerens et al. 2001). Incorporation of pest and disease resistance is an important component of white clover breeding programmes in New Zealand (Woodfield & Caradus 1996).

2.2.4 White clover nutritive value

The nutritive value of a feed is defined on the basis of nutrient concentration relative to animal requirements, or animal production response per unit of intake (Ulyatt 1981a). White clover herbage is considered the best quality component of grazed pastures because of its high nutritive value (Ulyatt 1981b). White clover has higher concentrations of crude protein (nitrogen x 6.25) and minerals, and lower concentrations of structural carbohydrates (cell wall) compared with other legumes and grasses (Ulyatt et al. 1977; Thomson et al. 1985; Minson 1990) (Table 2.1). Cell wall material (cellulose, hemicellulose and lignin) is slower and more difficult for ruminants to digest than the cell contents. The proportion of cell contents to cell wall material in white clover is high (Wilman & Altimimi 1984), resulting in rapid and high digestibility. Lignin, the indigestible portion of cell wall, is higher in white clover than in ryegrasses

(Steg et al. 1994), accounting for the lower digestibility of clover cell walls (Wilman & Altimimi 1984).

Table 2.1 Chemical composition of white clover herbage and mixed perennial ryegrass/white clover pastures, and the recommended dietary concentrations for high producing pasture fed dairy cows. Units are g/kg of DM unless otherwise stated.

Constituent	White clover	Spring pasture	Leafy summer pasture	Dry summer pasture	Guidelines for dairy cows[1]
Crude protein	240-290[2]	180-350[1]	140-220[1]	90-140[1]	180 – 240[c]
NDF[a]	270-320[2]	350-450[1]	420-520[1]	520-650[1]	At least 350
CHO[b]	160-200[2]	70-250[1]	70-250[1]	70-150[1]	340-380[d]
ME (MJ/kg DM)	12[3]	11 –12[3]	10[3]	9[3]	> 10.5

[a]NDF (neutral detergent fibre; cell wall) is required to stimulate chewing and saliva production, but reduces digestibility.

[b]CHO = soluble carbohydrate.

[c]Lower values are for cows producing 20 kg milk/day, higher values for 30 kg milk/day.

[d]These values are based on cows fully fed total mixed rations. Requirements for pasture diets are lower because the majority of energy from pasture fed diets comes from fermentable fibre.

[1]Kolver 2000.

[2]Ulyatt & Egan 1972, Ulyatt & MacRae 1974, Ulyatt et al. 1982.

[3]Holmes et al. 2002b.

Leaves contain much less cell wall material, particularly lignin, and more protein and soluble carbohydrates than stem material from the same plant (Black 1990). Because of its stoloniferous habit, the harvested portion of white clover is mainly leaves (leaflets and petioles) and flowers (flower heads and peduncles), as stolons are normally below grazing height (Frame & Newbould 1986). Consequently, a high digestibility is maintained throughout the year, although the digestibility of white clover flowers may

be lower than that of grass leaves and stems (Frame & Newbould 1986). In contrast, the leaf to stem ratio of grasses and erect legumes decreases with maturity (Frame et al. 1998). This results in reduced digestibility, crude protein and metabolisable energy contents (ME; energy reaching the tissues in the form of absorbed nutrients, excludes losses in the faeces, urine and methane) and higher fibre concentrations.

There has been little research on the nutritive value of different plant parts of white clover, however, lower *in vitro* dry matter digestibility, protein concentrations and estimated metabolisable energy have been found in white clover flowers than in its leaves (Table 2.2; Gibb & Treacher 1983; Wilman & Altimimi 1984; Stockdale 1999). The lowest NDF concentrations are found in the leaflets of white clover (218-240 g/kg DM), with higher concentrations in the petioles (387-388 g/kg DM) (Wilman & Rezvani Moghaddam 1998). NDF increases in clover flowers with advancing maturity (Wilman & Altimimi 1984).

TABLE 2.2 *In vitro* digestibility and crude protein concentration in organs of white clover at different stages of maturity. Data are averaged over 2 years and expressed in g/kg of DM. Adapted from Wilman & Altimimi (1984).

Plant organ	Stage of maturity	Crude protein	Dry matter digestibility	Cell wall digestibility
Leaflet	Flower bud	278	898	544
	Near full flower	293	890	503
	Seeds formed	204	883	515
Petiole	Flower bud	131	875	627
	Near full flower	100	840	561
Flower head	Near full flower	199	791	212
	Seeds formed	204	753	443
Whole flower	Near full flower	123	728	387
	Seeds formed	71	607	296

2.2.5 Ruminant feed digestion

Feed breakdown begins in the animal's mouth during chewing, where a large proportion of plant cells are ruptured enabling their contents to be released. The chewed feed is mixed with saliva, swallowed and then degraded by microbial digestion in the rumino-reticulum (rumen). The rumen is maintained at a temperature of 39°C, in anaerobic conditions. Changes in pH are normally restricted (pH 5.8 to 6.8) by the buffering effect of saliva (Holmes et al. 2002b). Once particle size has been sufficiently reduced (able to pass a 2 mm screen; Waghorn 1986), the digesta may pass through the omasum into the acidic abomasum. Further feed digestion occurs in the small intestine enabling absorption of amino acids (Holmes et al. 2002b).

Carbohydrate digestion

Dietary soluble carbohydrates are rapidly degraded in the rumen. Cell wall cellulose and hemicellulose fractions are degraded by the rumen microflora, yielding volatile fatty acids (VFA), which are the main source of energy for the ruminant (Agriculture and Food Research Council 1998).

Lactating pasture-fed dairy cows have rumen VFA concentrations between 90 and 180 mM/L (de Veth & Kolver 2001). The VFA from pasture fed ruminants typically comprise of 60 to 72% acetate, 15 to 23% propionate, 12 to 18% butyrate, and 3-5% as the minor VFA; isobutyrate, valerate and isovalerate (Holmes et al. 2002b). The rate of feed breakdown and the proportions of individual VFA produced depend on the physical and chemical composition of the feed and the microbial populations in the rumen. Feeds with a high proportion of cell walls are broken down slowly, producing a high proportion of acetate. Feeds with a high proportion of soluble carbohydrates are fermented rapidly, and produce a higher proportion of propionate and butyrate (Holmes et al. 2002b).

The energy released by metabolising VFA is greatest for butyrate, followed by propionate, then acetate, with the VFA produced from amino acids yielding the least energy (Holmes et al. 2002b). VFA are absorbed through the rumen wall, with a high proportion of butyrate converted to ß-hydroxybutyrate. Acetate and ß-hydroxybutyrate are major precursors for fat synthesis (Holmes et al. 2002b). Propionate is absorbed and

is a precursor for glucose, which in turn is the main precursor for milk lactose (Holmes et al. 2002b,c).

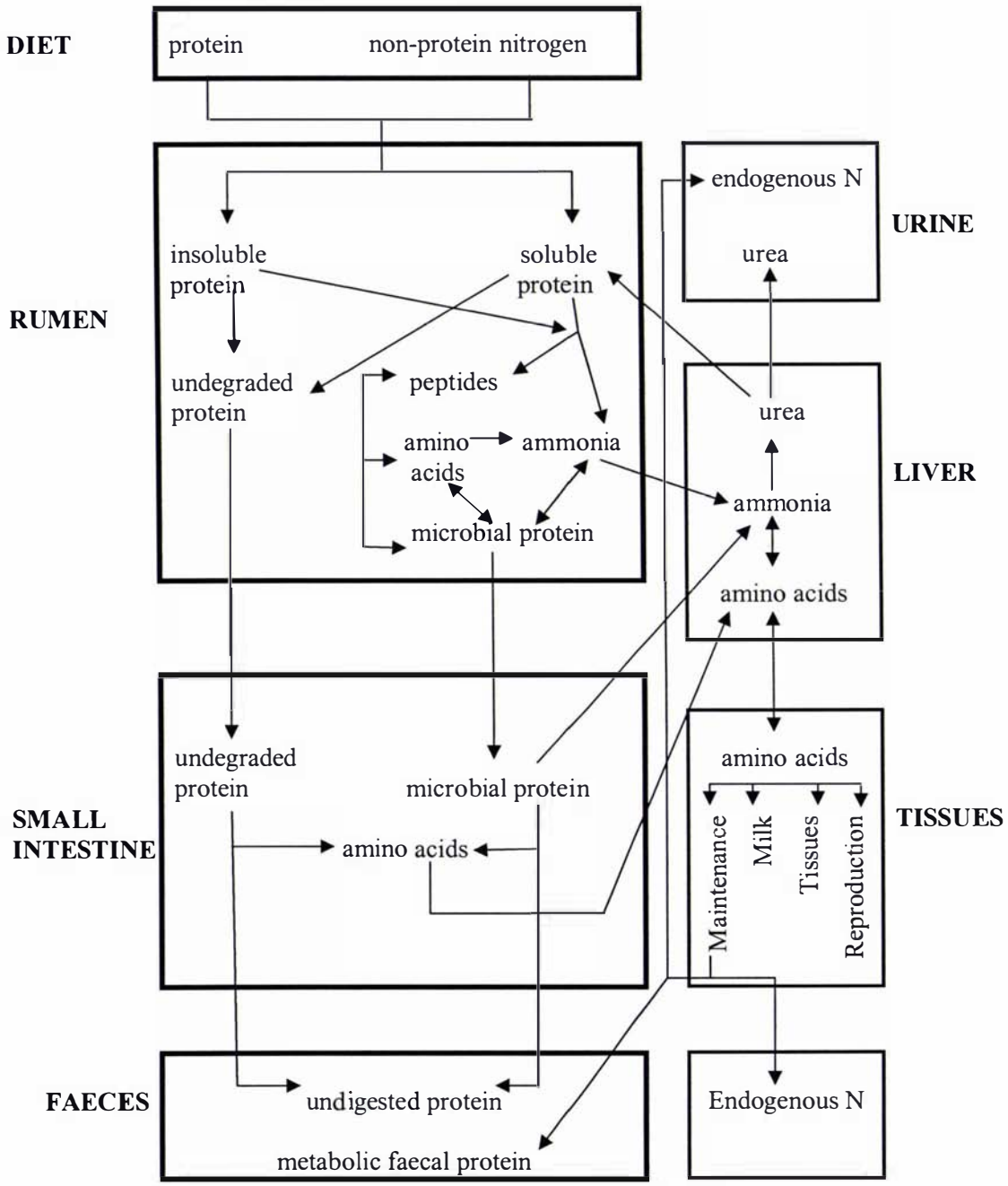
Protein digestion

The release of plant protein in the rumen is determined by the protein content of the feed, the resistance of plant cells to chewing, the degree to which it is released by chewing, and microbial degradation. The rate of protein degradation in fresh forages is determined by the rate at which it is accessed by rumen microbes. In general, soluble proteins are more rapidly and completely degradable than insoluble protein (Chalupa 1984). Ruminal protein degradation is reduced by any factor that slows release from the plant, reduces solubility or microbial protease activity or reduces the time forages spend in the rumen (Minson 1990). In pasture diets, approximately 70% of protein is degraded in the rumen (Waghorn & Barry 1987).

Rumen microbes hydrolyse protein to amino acids, some of which are directly incorporated into microbial protein, but most are further degraded to form ammonia (NH_3). When dietary protein is in excess of animal requirements amino acids may be degraded to form the VFA isobutyrate, valerate and isovalerate (van Soest 1982). Isobutyrate and isovalerate are required by fibre-degrading bacteria for growth and fibrolytic activity (Hungate 1966). The majority of rumen bacteria use ammonia as a nitrogen source (Bryant & Robinson 1962) for protein synthesis and rumen concentrations lower than 50 mg NH_3/L can reduce microbial growth rates (Satter & Slyter 1974). Microbial protein synthesis is more often limited by the energy supply than protein availability, except when the diet contains very little nitrogen or when the protein is protected from rumen degradation (Holmes et al. 2002b).

In addition to incorporation into microbial protein, there can be substantial ammonia-nitrogen absorbtion through the rumen wall (MacRae & Ulyatt 1974) and conversion to urea in the liver for excretion or recycling (Figure 2.2). Some of this urea will be recycled into the rumen via saliva, but the majority is excreted in urine (Holmes et al. 2002b).

FIGURE 2.2 Protein digestion and metabolism in lactating dairy cattle. Adapted from Chalupa (1984).



Rumen microbes are the major source of protein available to ruminants, followed by undegraded plant protein, and endogenous protein sloughed from the walls of the rumen (National Research Council 2001). The protein is degraded to amino acids in the abomasum and small intestine, which are then absorbed through the small intestine (Holmes et al. 2002b). Microbial crude protein has a lower nutritive value for

ruminants than plant crude protein because it contains a higher proportion (0.10 to 0.20) of nucleic acids (National Research Council 1985) as opposed to amino acids, and the mixture of amino acids is less suited to milk and meat production.

2.2.6 Performance of ruminants fed white clover

Intake

White clover has a lower resistance to chewing than grasses because of its lower cell wall content and shorter fibres (Minson 1990), resulting in a rapid release of cell contents and break down of feed particles. The high ratio of soluble to structural carbohydrate in white clover herbage leads to rapid fermentation, producing high concentrations of VFA and a rapid passage through the rumen (Ulyatt 1969). This rapid digestion results in higher feed intakes for animals with a high nutrient demand. Thomson (1984) summarised voluntary intake studies that compared white clover with perennial ryegrass fed fresh, dried or as hay or silage to sheep, young cattle and lactating dairy cows. Intakes of white clover were consistently 20% higher than perennial ryegrass.

Rumen protein degradation

Chewing of white clover during eating by cows releases 28 to 37% of its crude protein (Cohen & Doyle 2001), which is then rapidly degraded. The rate of DM degradation in the rumen of cattle, and protein loss is greater for white clover than for ryegrass. When Beever et al. (1986) fed either fresh perennial ryegrass or white clover to cattle they observed DM degradation rates of 14%/hour with the clover diet compared with 7%/hour for ryegrass. The high crude protein content of the clover herbage compared with ryegrass also resulted in high rumen ammonia concentrations. Despite a 90% increase in dietary crude protein intake with clover versus ryegrass, the average duodenal non-ammonia nitrogen supply was only 15% higher when the cattle were offered clover, indicating a large wastage of protein from clover diets.

Liveweight gain

Liveweight gains of ruminants fed white clover are consistently higher than those fed perennial (Ulyatt 1981b) or hybrid ryegrass (*Lolium* x *hybridum*) (Ulyatt 1970a), lucerne (*Medicago sativa* L.) or lotus major (*Lotus pedunculatus* Cav.) (Ulyatt et al.

1977). The rapid fermentation and high nutritive value of white clover enables ruminants to utilise white clover more efficiently than ryegrass for energy and liveweight gain (Rattray & Joyce 1974). A summary of experiments comparing animal production from white clover and grasses showed an average superiority of white clover for liveweight gain to be 65% for lambs and 30% for beef cattle (Ayres & Poppi 1993).

Milk production

Higher intakes are possible when cows are fed white clover rather than perennial ryegrass, contributing to higher milk yields. In an Australian study, Friesian cows in early lactation fed *ad libitum* white clover consumed 33% more DM than cows fed perennial ryegrass, and produced 25% more milk, 33% more milk fat and 38% more milk protein (Rogers et al. 1982). Over an entire lactation, cows grazing white clover had 21% higher milk yields than cows grazing perennial ryegrass, with the greatest differences from late spring to the end of summer, when the nutritive value of ryegrass was low (Rogers & Robinson 1984). When white clover and perennial ryegrass were fed at similar DM intakes milk production was still higher from cows fed white clover (Rogers et al. 1982; Johnson & Thomson 1996), indicating a higher conversion efficiency for clover.

Despite high milk yields from cows fed white clover, there is an energetic cost for ruminants metabolising high protein feeds, which lowers potential milk production. Cohen (2001) estimated ruminal losses of nitrogen from cows grazing white clover to be from 6 to 23% of nitrogen intake. If the energy used to excrete this nitrogen was instead used for milk production, an extra 0.5 to 2.0 kg milk may have been produced per cow per day.

2.2.7 Pasture bloat

Definition of bloat

Pasture bloat is a serious disorder of ruminants caused by retention of gas in the rumen. The gas produced by microbial fermentation in the rumen and by the release of carbon dioxide from salivary bicarbonate is normally eliminated by eructation (passage via the oesophagus), with some absorption through the rumen wall and passage via the abomasum. Bloat develops when gas is trapped within the rumen in a stable foam.

Foaming of the digesta is common; normally, however, the foam has a low persistence and low volume. During bloat, the foam is stable, persistent and accumulates in large amounts (Jones & Lyttleton 1969). This foam prevents normal expulsion of rumen gases, and as a consequence ruminal volume and intraruminal pressure increase. In severe cases death occurs due to cardiac or respiratory failure, sometimes within an hour of the commencement of feeding (Clarke & Reid 1974). It has been estimated that bloat costs the New Zealand dairy industry $25-50 million annually in animal deaths, labour, treatment costs and lost production (Morris et al. 1997).

Causes of bloat

Pasture bloat arises from the interaction of plant, animal, rumen micro-organisms and climatic factors contributing to gas and foam formation (Clarke & Reid 1974) and is most prevalent with immature pasture, particularly when containing legumes. The major plant species contributing to pasture bloat in cattle include the legumes white clover, red clover (*Trifolium pratense* L.) and lucerne, but excludes legumes containing moderate to high concentrations of condensed tannins (Kendall 1966). Bloat is predominant in spring and autumn, although it can occur throughout the year, on mature pastures, and on pastures without legumes (Clarke & Reid 1974).

Proteins in plants, saliva, and rumen microorganisms are the major foaming agents in the rumen (Carruthers 1986). Correlations have been reported between the incidence of bloat in cows fed lucerne with total nitrogen (Miltimore et al. 1964), soluble nitrogen, soluble protein nitrogen (Howarth et al. 1977), and total and soluble leaf chloroplast protein concentrations (Stifel et al. 1968).

There is a wide range of susceptibility to bloat between animals (Piper 1973). Possible animal factors affecting susceptibility listed by Mendel & Boda (1961) are "salivary flow and composition, fluid transfer from the rumen, rumen motility, gas production, microbial population, rate of eating, rumen pH and breakdown products of nitrogen metabolism (especially soluble protein)".

Prevention of bloat

Cattle and pasture management, as well as the use of surfactants and feed additives can help reduce the incidence and severity of bloat. Ensuring a continuous feed supply

promotes continuous and rapid rumen clearance (Majak et al. 1995) and reduces the potential production of carbon dioxide from acidification of rumen liquor (Waghorn 1991), thereby reducing the likelihood of bloat.

A wide variety of antifoaming agents have been used successfully to prevent bloat, including oils, synthetic detergents and other surface-active compounds (Clarke & Reid 1974). These compounds reduce the surface tension of the foam, decrease foam formation and release gasses trapped in foam. The administration of ionophore antibiotics such as monensin alter rumen fermentation, offering some degree of bloat protection (Hall et al. 2001). Adequate protection from bloat requires an effective concentration of the bloat-preventing agent in the rumen throughout the danger period. With the continual turnover of rumen contents, regular dosing is necessary to maintain sufficient concentrations of bloat preventative agents, which has associated labour and material costs.

Generally, bloat safe pastures have a lower digestibility and lower nutritive value than those that cause bloat. Feeding of roughage supplements such as maize (*Zea mays* L.) silage before grazing legumes can reduce, but not eliminate bloat (Stockdale 1994; Bretschneider et al. 2001). The risk of bloat is also reduced once legumes begin flowering (Majak et al. 1995), or if they contain sufficient concentrations of condensed tannins (Kendall 1966).

2.2.8 Perennial ryegrass/white clover association

Pure swards of white clover are impractical on farms because of low dry matter production, problems with pasture bloat, and the difficulty of maintaining them weed free. Agriculture in New Zealand is based predominantly on mixed pastures of perennial ryegrass and white clover. Such pastures produce about 15 t DM/ha/year (Hodgson 1989) compared to maximum DM yields in white clover monocultures of only 10-12 t/ha/year (Brock 1973).

White clover benefits mixed swards through its ability to fix up to 400 kg N/ha/year (Crush 1987), reducing the need for nitrogen fertiliser. The growth pattern of white clover also complements that of perennial ryegrass, as maximum clover production is in

late spring and summer (Harris 1987), helping to sustain herbage production and quality after the spring peak in ryegrass growth.

Although white clover has a higher nutritive value than perennial ryegrass, pure white clover diets contain excess protein. Harris et al. (1997) proposed that 50 to 65% of DM intake as white clover was optimal for milk production. Because of lower herbage yields from white clover than from ryegrass, lower clover contents are recommended to balance feed quality with feed supply. A clover content of at least 30% is required to significantly improve pasture nitrogen economy, forage quality and animal performance (Stewart 1984; Thomson 1984). However, dairy pastures in New Zealand have average clover contents of less than 20% of DM (Ettema & Ledgard 1992). Recent research has investigated the feasibility of growing ryegrass and white clover separately but allowing animals to select from both pasture species. Marotti et al. (2001) and Cosgrove et al. (2003) showed similar lamb liveweight gain and dairy cow milk production from separate swards to clover only diets, and better performance than from mixed swards or ryegrass only diets.

2.2.9 Protein limitations of mixed perennial ryegrass/white clover swards

New Zealand agriculture is based on year round grazing of temperate pasture. The protein and soluble carbohydrate concentrations of pasture vary throughout the year, so the diet is not always well balanced for animal production (Ulyatt 1997). Pasture protein is 75-80% soluble (Mangan 1982) and is rapidly and extensively degraded when it is released in the rumen (MacRae & Ulyatt 1974). Approximately 90% of soluble protein can be degraded within one hour of eating (Ulyatt 1997), and up to 50% of dietary protein can be degraded to ammonia and absorbed into the blood stream (Thomson 1982).

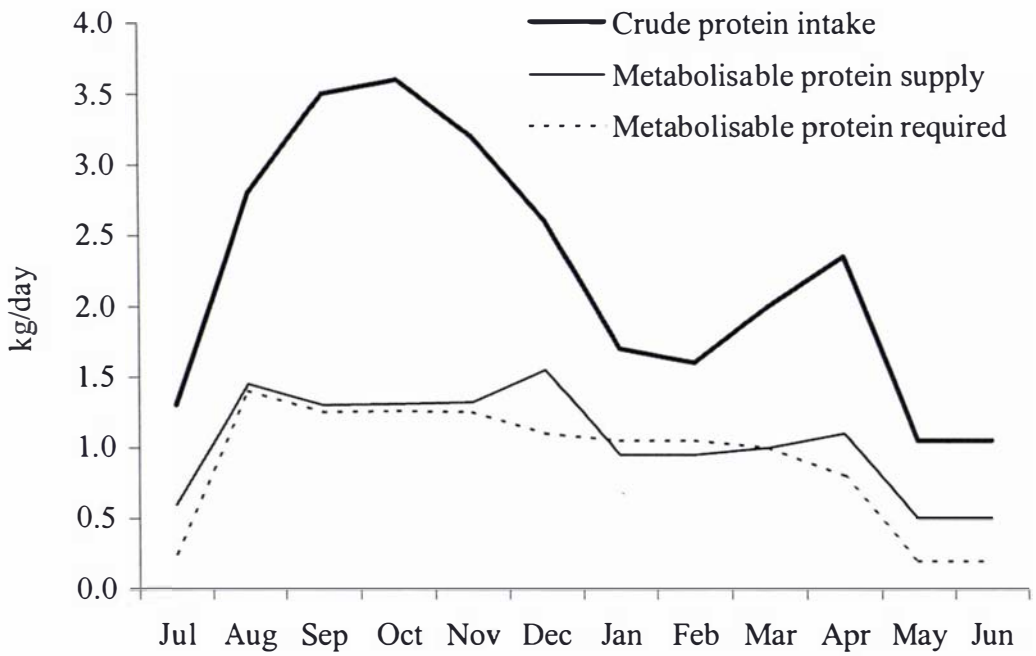
Absorbed ammonia is converted to urea primarily in the liver at an energetic cost of at least 15 kJ/g N (Martin & Blaxter 1965; Twigge & van Gils 1984), or up to 4% of ME intake (Waghorn & Wolff 1984). This reduces the available energy for animal production. Suggestions that conversion of ammonia to urea also requires substantial catabolism of absorbed amino acids (Beever 1993), have received less support in more recent studies with sheep (Milano et al. 2000) and lactating cows (Lapierre et al. 2004).

The National Research Council (1989) suggest crude protein (CP) concentrations for cows producing 13 kg milk/day of 150 g/kg of DM intake, increasing to 180 g/kg of DM intake at milk yields of 33 kg/cow/day. These data are based on mixed ration diets. Kolver (2000) recommended CP concentrations for pasture fed dairy cows of 180 g/kg DM for cows producing 20 kg milk/day, rising to 240 g CP/kg DM at production of 30 kg milk/day. When dietary crude protein concentrations are below these recommendations insufficient microbial protein will be formed, but at higher concentrations excess ammonia production results in wastage of protein and energy. The impact of a high CP solubility in New Zealand dairy systems is illustrated in Figure 2.3. The crude protein intake is high compared to the amount digested and absorbed, demonstrating the inefficiency of protein utilisation. Metabolisable protein supply is usually in excess of requirements, but may be below requirements in summer.

Spring and autumn dairy pasture is predominantly highly digestible green leaf, which is rapidly degraded and can lead to pasture bloat. Soluble carbohydrate concentrations (80-150 g/kg of DM) are low compared to crude protein concentrations (200-300 g/kg of DM), resulting in substantial protein wastage (Moller et al. 1996). When diets contain high concentrations of rapidly degradable protein, energy requirements are high to maximise incorporation of rumen ammonia into microbial protein. Reducing ruminal protein degradation would increase amino acid flow to the intestines and reduce microbial energy requirements. This can be achieved by protecting the protein from degradation in the rumen, or increasing passage rate through the rumen (Agriculture and Food Research Council 1998).

Summer pastures contain a high proportion of seedhead, stem and dead matter relative to leaf (Waghorn & Barry 1987). High fibre concentrations (Wilson & Moller 1993) are associated with lower crude protein concentrations, which may drop below 150 g/kg of DM (Moller et al. 1996). The slow fibre digestion and low crude protein concentration of mature pastures limits energy and protein intakes, and animal performance can only be sustained by substitution with rapidly digested forages with an adequate protein supply (Burke et al. 2002). Incorporation of condensed tannins into pasture diets can benefit animal production by reducing ammonia losses and increasing the supply of amino acids to the intestines (Waghorn et al. 1999).

FIGURE 2.3 Simulation of the protein supply and requirements of dairy cows grazing pasture in the Waikato region of New Zealand, using the feedTECH model. Adapted from Ulyatt (1997).



2.3 EFFECTS OF CONDENSED TANNINS ON RUMINANT PERFORMANCE

2.3.1 Condensed tannins

Condensed tannins (CT; also known as proanthocyanidins) are polyphenolic secondary plant metabolites, usually of high molecular mass, capable of complexing with proteins or carbohydrates (Ayres et al. 1997). CT are contained within the vacuoles or cell walls of plant cells, and can be expressed in various organs including leaves, stems, bark, roots, flowers or seeds (Barry 1989), depending on the plant species. CT are generally present in higher concentrations in reproductive than vegetative structures in temperate legumes (Terrill et al. 1992a).

The function of CT in plants is uncertain, but it has been proposed that some plants evolved CT production as a chemical defence against invasion by pathogens, or consumption by insects or herbivores (Swain 1979). At high concentrations, CT generally reduce plant nutritional value for ruminants, especially when dietary CP concentrations are low (Kumar & Singh 1984). Other perceived functions include allelopathy, protection from disease or UV light, healing after physical damage (Reed 1997), attraction of pollinators to flowers bearing anthocyanidins, or that they result from the shunting of primary metabolites when conditions are not conducive to growth (McMahon et al. 2000).

2.3.2 Distribution and concentration of condensed tannins in plants

CT are widespread in vascular plants. They may occur in trace amounts (<5 g/kg of DM) in grasses such as summer grass (*Digitaria sanguinalis* L.) and Yorkshire fog (*Holcus lanatus* L.) (Jackson et al. 1996b), but have higher concentrations in dicotyledenous plants (Table 2.3), including forage legumes such as lotus major (Jones et al. 1976) and sulla (*Hedysarum coronarium* L.) (Bate-Smith 1973). CT may be produced in high concentrations (>200 g/kg DM) in plants originating from warmer climates (Lowry et al. 1996), and are widespread in tropical trees, shrubs and herbaceous plants (Kumar & Vaithiyanathan 1990; Rittner & Reed 1992; Jackson et al. 1996a). In tropical environments, CT are normally regarded as anti-nutritional.

TABLE 2.3 Condensed tannin (CT) concentration of a range of forage species measured by the butanol-HCl method. Adapted from Terrill et al. (1992a).

Forage	g CT/kg DM
Legumes	
Erect dorycnium (*Dorycnium rectum* L.)	143
Lotus major (*Lotus pedunculatus* Cav.)	77
Sulla (*Hedysarum coronarium* L.)	45
Birdsfoot trefoil (*Lotus corniculatus* L.)	21
Perennial lupin (*Lupinus polyphyllus* L.)	1.7
Cicer milkvetch (*Astragalus cicer* L.)	1.6
Herbs	
Chicory (*Cichorium intybus* L.)	4.2
Sheep's burnet (*Sanguisorba minor* Scop.ssp. *muricata*)	3.4
Grasses	
Yorkshire fog (*Holcus lanatus* L.)	1.6
Perennial ryegrass (*Lolium perenne* L.)	1.1

The concentration of CT in a plant is controlled primarily by genetics, with secondary control exerted by the environment (Miller & Ehlke 1997). Barry & Forss (1983) found CT contents in lotus major grown in low fertility acid soils were halved when fertilised with phosphorus and sulphur. The CT concentration in the leaves of birdsfoot trefoil increased when fertilised with nitrogen (Briggs 1990), and when the temperature was reduced to below optimum for growth (Carter et al. 1999). However, responses to the environment vary between plant species. Moisture stress elevated CT concentrations in lotus major, but caused either no response or a decrease in CT concentrations in birdsfoot trefoil (Anuraga et al. 1993; Carter et al. 1999).

The leaves of some African trees increase CT content in response to browsing or may show diurnal variation (Furstenburg & van Hoven 1994). Season and stage of plant development also cause variation in CT concentration in some plant species (Bell et al. 1992; Joseph et al. 1998). CT increases in sainfoin (*Onobrychis viciifolia* Scop.) leaves

until maturity, then decline during senescence (Lees et al. 1995). In contrast, Iason et al. (1995) found the concentration of unbound CT in Yorkshire fog decreased with increasing leaf maturity, but fibre and protein bound CT concentrations increased. The highest total CT concentrations (2.8 g/kg DM) were found in dead leaves.

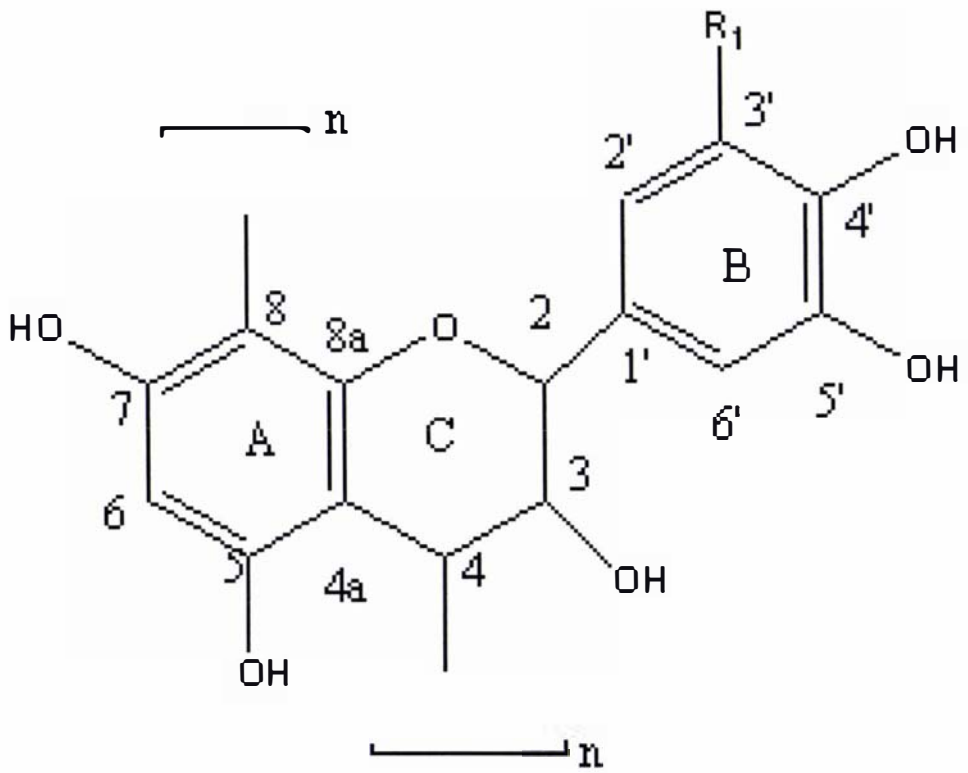
2.3.3 Chemistry of condensed tannins

Condensed tannins are released from the vacuoles of plant cells when chewed, enabling them to complex with plant proteins, primarily by hydrogen bonding (Loomis & Battaile 1966), forming an insoluble complex. Binding affinities differ with the type of protein and CT (Hagerman & Butler 1981). Condensed tannins may also bind directly to carbohydrates, but usually with a lower affinity than for protein (Barry 1989).

The monomer units of CT (Figure 2.4) may be classified according to the number of hydroxyl groups on the B-ring; procyanidins have a dihydroxy B-ring, and prodelphinidins have a trihydroxy B-ring (Kraus et al. 2003). Their activity is further affected by the stereochemistry of the C-rings determining the final shape of the polymer (Barry & McNabb 1999).

The binding capacity of CT depends on their hydroxylation pattern (Reed 1995), types of terminal groups (Foo et al. 1996), the structure of binding sites (Asquith et al. 1987), and polymer size (Hagerman & Butler 1981). CT with higher degrees of polymerisation (Horigome et al. 1988) and higher proportions of prodelphinidin compared to procyanidin units (Jones et al. 1976) correlate positively with protein precipitation. The reactivity of CT is also pH dependent; protein-CT complexes are stable and insoluble at pH 3.5-7.0, but dissociate and release protein at pH < 3.5 and > 8.5 (Jones & Mangan 1977).

FIGURE 2.4 Chemical structure of a condensed tannin monomer, with n representing binding sites for additional monomers. When R_1 on the B-ring is H the monomer is a procyanidin, when R_1 is OH the monomer is a prodelphinidin. From Barry & McNabb (1999).



2.3.4 Effect of condensed tannins on protein digestion

CT can have large effects on the digestion and absorption of nutrients, but they do not appear to be digested (Terrill et al. 1994). The binding of CT to plant proteins reduces protein solubility (Jones & Mangan 1977) and degradation to ammonia in the rumen (Barry et al. 1986; Chiquette et al. 1989), increasing dietary protein flow to the intestines. CT reduce populations of some proteolytic bacteria *in vivo* (Min et al. 2002). Most CT is bound to protein in the rumen (pH 5.5-7.0), with some dissociation in the abomasum (pH 1.3-3.0) (Waghorn et al. 1990) enabling hydrolysis and absorption of amino acids from the intestines. The quantity of non-ammonia nitrogen and amino acids reaching the small intestine is normally increased by CT, provided microbial growth is not inhibited (Waghorn et al. 1987a; Waghorn & McNabb 2003).

Low dietary concentrations of CT (below 50 g/kg DM) may reduce rumen ammonia concentrations by 20 to 40%, but medium concentrations (50-120 g/kg DM) can cause a 70% reduction in rumen ammonia concentration (Waghorn et al. 1999). Effects of CT on rumen microbial protein synthesis appear to be minor with temperate forage legumes unless CT concentrations are high (over 120 g/kg DM) (Barry et al. 2001). When birdsfoot trefoil contained 22 g CT/kg DM the flow of essential amino acids to the small intestine in sheep was increased by 50% (Waghorn et al. 1987b).

The increased flow of amino acids to the small intestine due to CT does not always result in an increase in the amount of amino acids absorbed. Waghorn et al. (1994a) reported that the CT in lotus major (55 g/kg DM) increased the flow of amino acids to the intestine, but the fractional absorption of amino acids was reduced, resulting in negligible effects on overall protein nutrition.

Wang et al. (1996c) showed the CT in birdsfoot trefoil slowed absorption of the essential amino acids methionine and cysteine, so that less were absorbed in the first half of the small intestine and more in the second half, relative to when CT were deactivated. Plants containing higher concentrations or more astringent CT (for example, lotus major, mulga (*Acacia aneura* Benth.) and carob (*Ceratonia silique* L.)) may slow absorption, inhibiting the net uptake of amino acids. Inhibition of absorption is probably due to a lack of dissociation of CT and plant proteins, or to effects of CT on the intestinal mucosa (Waghorn et al. 1999).

2.3.5 Relationship between condensed tannin concentration and ruminant nutrition

CT may have either anti-nutritional or beneficial effects on ruminants. The balance between dietary CP concentration and the binding capacity of CT determine overall effects on nutrition. Diets with a low protein and high CT concentration are likely to have detrimental effects on animals.

Studies with temperate legumes indicate that CT concentrations should be in the range of 20 to 40 g/kg DM consumed for optimal effects on ruminant digestion and production (Waghorn et al. 1990), and concentrations greater than 60 g/kg DM are likely to be detrimental (Waghorn et al. 1998). However, the optimum concentration varies across plant species, as some CT are more astringent (Mangan 1988) and detrimental to digestion than others (Waghorn et al. 1998). The biological effects of CT depend on the chemical structure and concentration of the CT, and the concentration of protein and other constituents in the diet. Sainfoin, containing CT concentrations as high as 80 g/kg DM, has been shown to be of high nutritive value for sheep (Waghorn & McNabb 2003), whereas sheep growth rates were severely depressed with a diet containing carob pulp with only 25 g CT/kg DM (Priolo et al. 2000).

The response to CT also varies between ruminant species. Some animals, such as deer, produce proline-rich salivary proteins which bind with and appear to lessen the impact of CT on digestion (Austin et al. 1989). Domestic sheep and cattle do not produce these proteins in their saliva (Austin et al. 1989). Establishing relationships between CT concentration and animal performance is also complicated by variations in CT analytical techniques (Section 2.5.4) across experiments.

2.3.6 Effect of condensed tannins on fibre digestion and voluntary feed intake

The negative effects of high dietary CT concentrations may be due to inhibition of rumen microbial enzymes, and binding with lignocellulose may reduce microbial attachment (Jones et al. 1994) and degradation (McSweeney et al. 2001). Min et al. (2003) suggested an association of CT with bacterial extracellular fibrolytic enzymes, inhibiting their activity, whilst McAllister et al. (1994) showed that CT from birdsfoot trefoil can also cause detachment of *Fibrobacter succinogenes* from cellulose fibres. High concentrations of CT in lotus major (63 and 106 g/kg DM) depresses rumen fibre

digestion (Barry & Manley 1984; Barry et al. 1986), which can reduce the energy value of the forage (McMahon et al. 2000) and lower voluntary feed intake (VFI) because rumen clearance rates are reduced (Waghorn et al. 1994b).

Molar ratios of the principal VFA's (actetate, butyrate, propionate) are not usually affected by CT, but their concentrations in rumen liquor are often reduced (Waghorn et al. 1999). This suggests either a reduced rate of degradation or a larger rumen pool size when CT are present in the diet. Barahona et al. (2003) reported an inverse relationship between the concentration of the protein-derived branched-chain VFA (isobutyrate and isovalerate) and the concentration of CT in seven tropical legumes *in vitro*, with similar findings *in vivo* for CT-containing legumes (Waghorn et al. 1994b).

2.3.7 Effects of condensed tannins on milk production and composition

The New Zealand dairy industry relies on year-round grazing of temperate forages, principally mixed pastures of perennial ryegrass and white clover. Milk production declines rapidly in summer-autumn, partly due to declining feed quality and availability (Clark 1995), so a higher proportion of legumes in the diet could maintain cow performance and profitability.

Summer grazing experiments with cows fed birdsfoot trefoil have shown higher milk and milksolids (fat + protein) yields than for perennial ryegrass or white clover diets (Table 2.4; Harris et al. 1998). This was attributed to a higher feed quality and feed conversion efficiency. Milk protein concentrations were highest and milk fat concentrations were lowest in birdsfoot trefoil diets.

Few studies have evaluated the effects of CT in forage legumes on milk production and composition (Table 2.4). Recent research has found positive effects of CT in birdsfoot trefoil on milk production and composition in sheep and cattle. In a study with sheep grazing birdsfoot trefoil (Wang et al. 1996a) the CT (40 g CT/kg DM) did not affect milk yield or composition at peak lactation, but from mid to late lactation it increased milk, protein and lactose yields by 21, 14 and 12%, respectively. The CT did not affect milk fat yield, but its concentration was decreased whilst lactose % increased. These results were attributed to increased essential amino acid absorption from the small

intestine and a reduction in energy expenditure for urea synthesis and methane production (Waghorn et al. 1998).

Experiments in which Friesian dairy cows in both mid (Woodward et al. 2000) and late lactation (Woodward et al. 1999; Woodward et al. 2004) were fed birdsfoot trefoil (25, 27 and 26 g CT/kg DM, respectively) showed CT increased milk yields by 14, 20 and 10%, respectively, with no effects of CT on feed intakes. This suggests that milk yields were increased due to increased feed conversion efficiency. Milk protein concentrations were increased by 5-6% due to CT, but there were no significant effects on milk fat.

Other diets containing CT have not always benefited milk production. The condensed tannins in sulla (44 g/kg DM) did not benefit yield or fat % in sheep milk in mid to late lactation, but altered the concentrations of some fatty acids (Roy et al. 2002). Milk protein and lactose were not reported. The effects of CT in sulla on milk production in dairy cows have not been quantified, but pasture-fed cows supplemented with sulla silage had similar milk yields and milk protein concentrations to those fed other silages with no CT (Table 2.4). However, the CT concentrations of sulla in that experiment were low (16 g/kg DM). Cows fed sulla containing 27 g CT/kg had higher milk protein concentrations than those fed perennial ryegrass, and produced 35% more milk (Woodward et al. 2002b). This is likely to be from a combination of higher feed intakes and improved feed quality.

Few experiments have reported the effects of supplementing CT-containing plant extracts or by-products on milk production. Bhatta et al. (2000) found no effect of tamarind (*Tamarindus indica* L.) seed husks (up to 7.4 g CT/kg dietary DM) on total milksolids yield or the yield of milk components, but reported a small increase in milk protein % with *Bos indicas* x *Bos taurus* cows. Mimosa (*Acacia spp*) bark extract (4.1 g CT/kg dietary DM) fed with pasture did not affect milk yield or composition of Friesian cows (Mashudi et al. 1997).

TABLE 2.4 Effects of diets containing condensed tannins on milk yield and composition of sheep or Friesian cows.

Feed/control	CT in diet (g/kg DM)	Animal	Milk response	Data source
Birdsfoot trefoil/white clover	25	Cows	11% increase in yield 8% decrease in fat %	Harris et al. (1998)
	22	Cows	8% increase in yield 4% increase in protein %	
Birdsfoot trefoil/+ PEG[a]	45	Sheep	Mid to late lactation: 21% increase in yield 14% increase in protein 12% increase in lactose	Wang et al. (1996a)
Birdsfoot trefoil/+ PEG[a]	27	Cows	20% increase in yield 5% increase in protein %	Woodward et al. (1999)
Birdsfoot trefoil/+ PEG[a]	26	Cows	10% increase in yield 6% increase in protein %	Woodward et al. (2004)
Birdsfoot trefoil/+ PEG[a]	25	Cows	14% increase in yield 6% increase in protein %	Woodward et al. (2000)
Sulla/+ PEG[a]	44	Sheep	No effect on milk or fat yield Increased concentration of some fatty acids	Roy et al. (2002)
Pasture with sulla silage or birdsfoot trefoil silage/Pasture with pasture silage or maize silage	16	Cows	Sulla: similar to pasture and maize silage	Woodward et al. (2002a)
	34		Birdsfoot trefoil: 15% increase in yield 15-16% increase in MS[b] 4-5% increase in protein	
Pasture and mimosa/Pasture	4	Cows	No effect on milk yield or composition	Mashudi et al. (1997)
Straw and compound feed mix and tamarind seed husk/ Straw and compound feed mix	7	Cows[c]	No effect on yield or fat 1% increase in protein %	Bhatta et al. (2000)

[a] PEG (polyethylene glycol) binds to and inactivates condensed tannins (see Section 2.5.6), [b] MS = milksolids (fat plus protein yield), [c]crossbred.

2.3.8 Liveweight gain and wool production

The effects of CT contained in forage legumes on liveweight gain and wool growth of sheep have shown mixed results (Table 2.5). Responses to CT are generally positive with birdsfoot trefoil containing up to 40 g/kg DM, but variable and often negative with other diets (Waghorn et al. 1998). The effects of different types of CT is highlighted by sulla CT concentrations as high as 88 g/kg DM which did not reduce liveweight gain or wool growth in young sheep (Douglas et al. 1999). In contrast, diets containing only 25 g carob CT/kg DM (Priolo et al. 2000) severely inhibited liveweight gain in young sheep.

2.3.9 Ruminant health and fertility

The use of CT-containing forages could reduce the need for proprietary drenches and greatly reduce labour costs to control bloat, internal parasites and fly strike. CT have long been known to prevent pasture bloat (Jones et al. 1973). The binding of CT to soluble protein in the rumen prevents the formation of stable foams that trap rumen gases. Sarkar et al. (1976) recognised the legumes sainfoin, birdsfoot trefoil and cicer milkvetch as bloat safe due to the presence of CT, and Tanner et al. (1995) demonstrated their ability to collapse protein foams in a dose dependent manner.

Li et al. (1996) estimated that the CT threshold for bloat safety in forage legumes is between 1 and 5 g/kg DM. However, this estimate has not been tested *in vivo* and some of their data were based on CT measured using the acidified vanillin technique, which accounts for only about half of the total CT in legumes (Terrill et al. 1992a). The CT required for bloat prevention will depend on the nutrition, hunger, rate of eating and diet composition (Waghorn & McNabb 2003), as well as the type and concentration in the diet.

TABLE 2.5 Effects of condensed tannins (CT) in lotus species and sulla on liveweight gain (LWG) and wool growth of sheep.

Feed	CT in diet (g/kg DM)	Animal	Production response	Data source
Birdsfoot trefoil	23	Young sheep	12% more wool No effect on LWG	Wang et al. (1994)
Birdsfoot trefoil	40	Young sheep	11% more wool	Wang et al. (1996b)
Birdsfoot trefoil	50	Young sheep	No effect on LWG or wool growth or carcass weight	Douglas et al. (1999)
Mix of birdsfoot trefoil and ryegrass	30	Young sheep	9% more LWG 5% more wool	Waghorn & Shelton (1995)
Lotus major	80	Sheep	7% less wool	Waghorn et al. (1994a)
Lotus major	100	Sheep	25% less LWG	Barry & Duncan (1984)
Sulla	40-50	Sheep	10% less LWG	Terrill et al. (1992b)
Sulla	88	Young sheep	No effect on LWG or wool growth 8% increase in carcass weight for high feed allowance no effect for low allowance	Douglas et al. (1999)

Control of internal parasites has relied heavily on the use of proprietary anthelmintics. Although these compounds have been successful, the development of resistance in some countries (Waller 1994), including New Zealand, indicates that their continued use is not sustainable. CT-containing diets fed to lambs with an intestinal worm burden have enabled good liveweight gain and wool growth, despite high faecal egg counts (Robertson et al. 1995). Sulla has reduced gastrointestinal worm numbers in sheep (Niezen et al. 1995) but no measurements have been undertaken with cattle. *In vitro* assays have demonstrated potent anthelmintic effects of CT extracted from a range of legumes (Molan et al. 2002).

Faecal contamination (dags) and ovine myasis (flystrike) are of major economic importance to the New Zealand sheep industry, causing loss of liveweight, death, and wool and pelt damage. Dags and flystrike have been reduced in sheep fed birdsfoot trefoil relative to sheep fed pasture (Leathwick & Atkinson 1995).

Ovulation rates in sheep can be increased by improving protein nutrition (Smith 1985). Experiments to determine the effects of CT in birdsfoot trefoil on ovulation rates have generally been positive (Luque et al. 2000; Min et al. 1999), depending on the condition of the ewe. The effects of CT on cattle fertility have not been studied, but any improvements in the protein-energy status should be beneficial.

2.3.10 Environmental effects of condensed tannins

Production of the greenhouse gases methane and nitrous oxide may be reduced by incorporating CT into the diet of ruminants. Waghorn et al. (2002) fed lotus major (53 g CT/kg DM) to sheep and reported that CT reduced methane production per unit dry matter intake by 16%. The CT in birdsfoot trefoil has also decreased methane emissions per unit feed intake by 13% in dairy cows (Woodward et al. 2004).

Urine patches are the dominant source of nitrous oxide emissions (de Klein et al. 2003) and of nitrogen leached from grazed pastures (Ryden et al. 1984). Nitrogen leached from agricultural systems can reduce groundwater and surface water quality. Dietary CT lowers urinary nitrogen excretion and this has potential for reducing nitrous oxide losses and nitrate leaching from dung and urine patches (Waghorn & McNabb 2003).

2.4 SOURCES OF CONDENSED TANNINS

2.4.1 Current sources of condensed tannins in New Zealand

A number of forage legumes containing CT have been investigated for use on New Zealand dairy and sheep farms. Birdsfoot trefoil and sulla are likely to be the most beneficial for dairying. However, they are not as tolerant to grazing and are less competitive in fertile soils than is white clover. DM yields of birdsfoot trefoil may be lower than for ryegrasses or lucerne and its quality declines when mature, and sulla is prone to crown damage (Waghorn et al. 1998).

Beneficial effects of mixing forages containing CT with those that do not can only be achieved if the CT content is sufficiently high to bind with proteins in the non-CT containing plant (Barry & McNabb 1999). Animal production has been improved when feeding the CT-containing legume lotus major with other forage species (Waghorn et al. 1998). However, this species is more competitive in cold, acidic, low fertility soils, and loses palatability when mature. The astringent nature of the CT in lotus major is demonstrated by reduced voluntary feed intake and protein absorption when fed as a sole diet (Waghorn et al. 1998).

2.4.2 Approaches to increasing condensed tannin production in white clover

Waghorn et al. (1990) suggested white clover as the ideal source of CT for New Zealand agriculture. New Zealand farmers are already familiar with the management of white clover, as opposed to other CT-containing species. Ease of management is important because it determines the extent to which farmers can capture the potential of improved cultivars, and also determines the requirements for labour, technical skills and additional inputs (Snaydon 1978). The genes for producing CT are present in white clover (Jones et al. 1976), but the concentrations must be increased to greater benefit ruminant performance.

Biotechnology

Waghorn et al. (1990) speculated that foliar CT concentrations of up to 80 g/kg DM may increase animal productivity by 10 to 15% and eliminate bloat when white clover accounts for 20-30% of the dry matter consumed. Several researchers are currently working towards understanding the genetic factors that regulate CT biosynthesis, with

the long term aim of optimising CT concentrations in specific tissues of several plant species (Morris & Robbins 1997).

Recent research has identified a gene encoding an enzyme that converts red plant pigments (anthocyanins) into epicatechin (one of the monomers of CT), enabling production of CT in tobacco (*Nicotiana tabacum* L.) leaves, which do not usually contain CT (Xie et al. 2003). This technology, along with other associated genes in the CT pathway, may be used to develop a white clover that expresses CT in its leaves (D. R. Woodfield personal communication).

Despite the potential benefits of biotechnology, the safety and environmental impact of genetically modified (GM) crops has been questioned, with New Zealand having one of the most stringent regulatory environments (Woodfield 2001). The regulations regarding testing of GM organisms will result in long delays before such plants can be released commercially. Lack of public acceptance of genetic manipulation may also limit potential opportunities.

Conventional plant breeding techniques

Conventional breeding and selection has formed the basis for improving forage quality, and selection for forage quality has long been recognised as achievable (Corkill 1958). Phenotypic recurrent selection has been the predominant breeding method for white clover (Williams 1987b). Repeated cycles of phenotypic evaluation, selection of superior plants and poly-crossing amongst selected plants is performed to increase the frequency of favourable alleles. Each cycle may take several years (Faville et al. 2003). Recent developments in genome mapping for white clover, and marker-assisted selection of desirable traits should accelerate traditional plant breeding programmes (Faville et al. 2003).

White clover expresses CT in its flowers, and in trace amounts in trichomes on the lower leaf surface (Woodfield et al. 1998). CT are evenly distributed through the petals with none in the calyx or peduncle. No CT is present in the anthers, but the filaments contain low concentrations, and the stigma and style contain high concentrations (Woodfield et al. 1998). Variation between genotypes in trichome density has been identified, but foliar CT concentrations are extremely low, ranging from 0.10 to 0.96 g

CT/kg DM (Woodfield et al. 1998). Higher concentrations of CT occur in the flowers of white clover (Jones et al. 1976). From 28 white clover cultivars grown in Palmerston North, New Zealand, floral CT concentrations ranged from 65 g/kg DM for Aran and Tillman through to 88 g/kg DM for Triffid and Prop (D. R. Woodfield, unpublished data).

Stockdale (1994) suggested that the CT in white clover flowers reduce the incidence of bloat in lactating dairy cows when flowers are more than 5% of the dry matter fed. However, no information is available concerning white clover CT concentrations and animal production, and seasonal effects on flowering will vary the dietary supply of CT. Increasing the availability of floral CT in white clover for grazing animals could involve phenotypic selection for one or more of the following:

- Increased floral CT concentration
- Increased flowering duration
- Increased flowering density (flowers/m^2)
- Increased flower head size

2.4.3 Flowering in white clover

White clover flowering intensity is determined by the number of potential flowering sites (stolon growing points), and the ability of these sites to respond to the flowering stimulus. The ability to flower depends on the plant changing from vegetative to reproductive growth, which is controlled by temperature and day length. There is considerable variation in flowering response between populations of different origins. Genotypes originating from high latitudes behave as long-day plants in their natural environment, initiating flowers as the photoperiod increases in spring. Genotypes of lower latitudes initiate flowering through autumn, winter and early spring, until day length and temperature increase (Thomas 1987). In New Zealand, white clover genotypes obtained from low latitudes initiate flowering from early autumn to spring, but genotypes from high latitudes initiate flowering later in winter and continue into summer (Thomas 1979; Williams 1987a).

Despite cultivar differences in the time of floral initiation, flowers appear at a similar time, as the rate of flower appearance depends on the rate of vegetative growth (Thomas

1961). A flower usually emerges from the surrounding sheath a few days after its subtending leaf emerges (Thomas 1980). Flowers initiated in early winter do not appear until spring because of slow growth in winter. Those initiated in late winter also appear in spring, when rapid growth begins (Williams 1987a).

In New Zealand, flower head emergence usually starts in late October and peaks in mid December, triggered by high temperature, day lengths greater than 12 hours, and high light intensity at the stolon level (Hill et al. 1999). Mediterranean (low latitude) plant material is slightly earlier flowering than that from northern Europe (high latitude) (Williams 1987a). Peak flowering dates vary by about five weeks between early and late flowering cultivars (Clifford & Baird 1993).

Under weak flowering stimuli the stolon growing point commonly initiates a sequence of one or two nodes bearing flowers, followed by one to three vegetative nodes, and this sequence is repeated several times (Thomas 1987). When the flowering stimulus is stronger, the ratio of flowering to non-flowering nodes is increased. This ratio is under genetic and environmental control (Thomas 1987), and is enhanced by high light intensity and good mineral nutrition (Haggar et al. 1963). In the field, there is usually a maximum of two to three flowers per stolon (Clifford 1985).

In mixed perennial ryegrass/white clover plot trials grazed by sheep in New Zealand, the average flower density between October and March for 110 white clover ecotypes and cultivars ranged from 16 to 95 flowers/m^2 (Woodfield & Caradus 1994). Avoiding moisture stress also allows more flowers to be produced (Hill et al. 1999). Defoliation also increases the number of sites for flower development. The second flush of flowering following peak initiation in spring is likely to be strongly stimulated by this means (Thomas 1987).

2.4.4 Breeding of white clover for increased floral condensed tannin concentrations

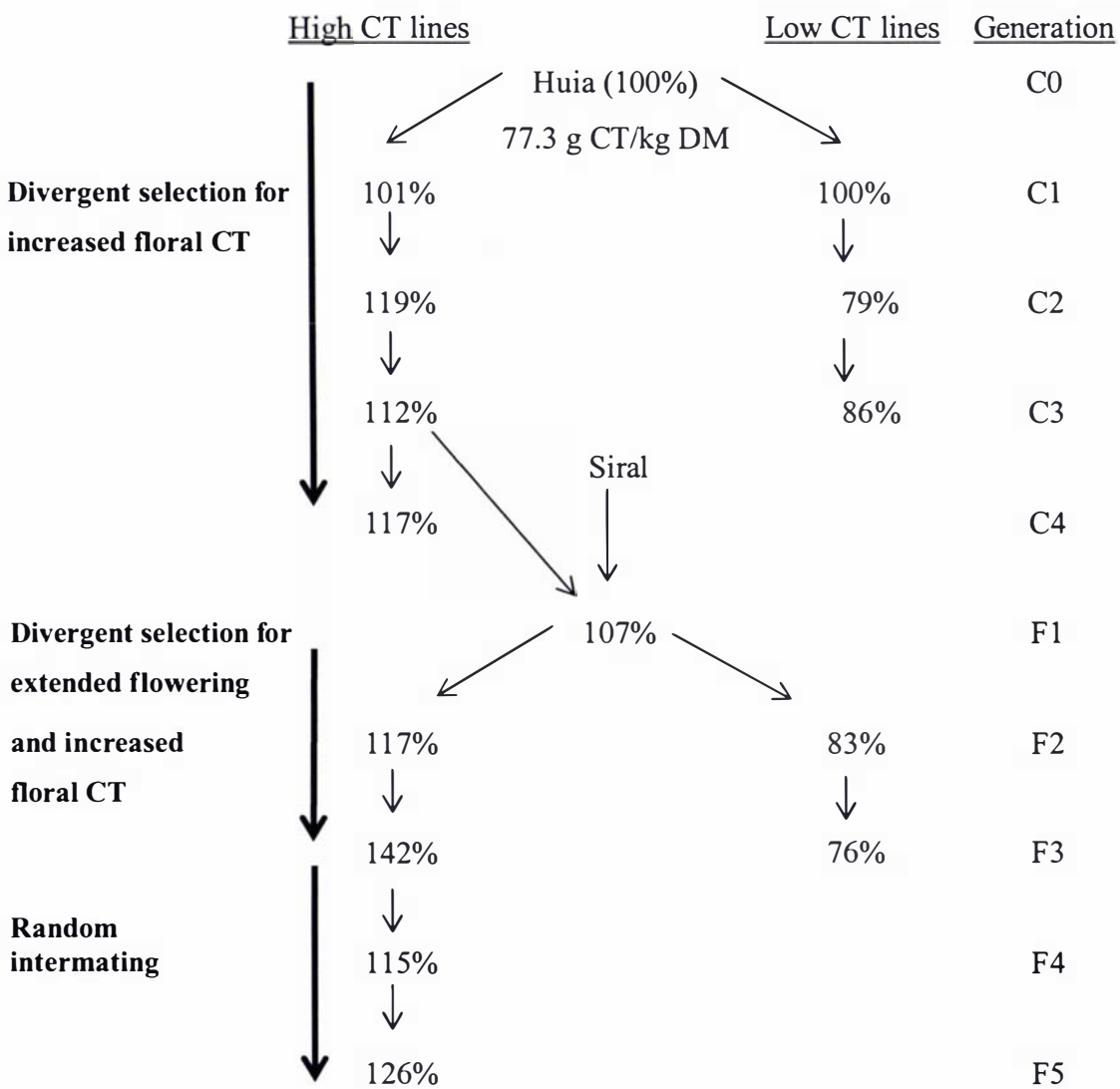
The first attempt to select for higher expression levels of floral condensed tannins in white clover was made at AgResearch Grasslands, New Zealand (Woodfield et al. 1998). Selection for HT (high tannin) white clover involved three phases: (i) divergent

selection for floral CT content; (ii) selection for extended flowering and high floral tannin content; and (iii) seed multiplication without selection (Figure 2.5).

In the first phase, four cycles of divergent selection for floral CT content were undertaken in Grasslands Huia white clover, resulting in development of high and low floral CT lines which differed by 66% in CT content (Woodfield et al. 1998). To make this CT available to grazing animals over a greater period of the year, phase two involved the high floral CT Huia selection being crossed with plants selected from the cultivar Siral for their extended flowering season. Siral is an Algerian ecotype of white clover developed in Australia.

Two cycles of selection for increased floral CT content and extended flowering were undertaken. The CT concentration in the resulting F3 generation was 32% higher than the mean of the two parental cultivars (Grasslands Huia and Siral). In phase three, two generations of random intermating without selection for CT content or flowering duration were undertaken under stringent isolation. The CT content of the resulting F5 generation (HT clover) was only 19% higher than the mean of the parental cultivars (D. R. Woodfield unpublished data) indicating that some of the selection gains had been lost during seed multiplication.

FIGURE 2.5 Breeding programme of HT white clover. Floral condensed tannin (CT) concentrations (measured by the Butanol-HCl technique of Terrill et al. (1992a) using birdsfoot trefoil CT as a standard) relative to that of Grasslands Huia white clover are presented for each generation. All CT analyses were performed on open florets of a similar age, excluding brown/senescing florets. C0 to C5 refers cycles 0 to 5 of selection, F1 to F5 refers to the first to fifth generation of hybrid offspring from crossing high CT Huia with Siral.



2.5 EVALUATION OF NEW FORAGE CULTIVARS CONTAINING CONDENSED TANNINS

2.5.1 Importance of the evaluation process

Before a new forage cultivar can be commercially released it should be thoroughly tested, preferably under grazing in similar field conditions to which it must perform (Morley 1978; Hodgson 1981; Ceccarelli et al. 1992). Breeding for improved forage quality without including animal evaluations of the new selections can lead to false conclusions (Vogel & Sleper 1994). Animal production data derived from new cultivars also have a role in technology transfer, usually leading to a more rapid adoption for commercial use (Laidlaw & Reed 1993). Differences in feeding value can be assessed by grazing trials, where a standard cultivar is included for comparison with the new forage, and where intake is not limited by herbage availability (Ulyatt 1981b). Careful administration of experiments should prevent interactions between the forage and its management that could mask or enhance actual differences that exist between treatments (Laidlaw & Reed 1993). However, animal performance experiments are only indicators of the potential value of the plant being assessed (Hodgson 1981), and should be complemented with agronomic evaluations and field trials that resemble commercial farm management practices.

2.5.2 Experimental design

Many forage species are grown in mixed swards, making it difficult to assign any measured response to a specific sward component. Use of pure swards avoids complications due to varying proportions of different plant species in treatment comparisons (Minson et al. 1960). However, differences in animal performance obtained by grazing pure swards may not occur with mixed swards (Snaydon 1978), due to either dilution of the active components or interactions with other sward components. Many forages are initially evaluated in monoculture, and if their performance is promising, further evaluation, including compatibility with companion species will be required.

New cultivars should be tested against the best alternative (control) cultivar. A control cultivar is one for which much information is already available; it is often used widely so that different experiments with the same control may be compared more reliably

(Thomas & Laidlaw 1981). Controls should be the most popular and the most promising alternative cultivar for the particular situation (Reed 1994).

The number of replicates required for an experiment requires establishing a balance between experimental costs (land area, number of animals, labour), the need to adequately account for variation (pasture composition, soil type, topography, animal variability) and the desired minimum detectable difference between the new cultivar and the control (Robards 1980).

2.5.3 Resources

Plant evaluation through animal production can provide extensive information, but will compromise between the desire to study a total system on a large scale and the availability of resources (Morley 1978). For example, CT affect many health and productivity parameters in animals, and measuring each of these would be both time-consuming, expensive and could require large numbers of animals. Because of financial and time constraints, few grazing experiments have evaluated all animal health and production parameters (Evans et al. 1979). It is important to measure the parameters most likely to have the greatest economic effect on the animal or system being studied. For example, the effects on milk production and bloat are important in dairy cows, whereas in sheep, measuring the effects on liveweight gain and worm burdens may be more useful. Time, personnel and resource requirements may become so large as the complexity of an experiment increases that the number of treatments that can be evaluated must be restricted (Morley 1978).

2.5.4 Plant performance

Desirable characteristics can be negatively correlated, so that selection for one characteristic may lead to unfavourable responses in another. This is the case in white clover, where selecting for increased flowering may reduce stolon branching (Thomas 1980). Where negative correlations exist, acceptable lower limits for the related characters need to be defined when selecting for the major variable (Clements 1970). The production of CT represents a significant metabolic cost to the plant (Chew & Rodman 1979). It is therefore important to determine the effect that the partitioning of some plant resources to CT production has on the growth and persistence of plants that

contain them, as the amount of herbage consumed is the major determinant of animal production (Buxton et al. 1996).

The response of animals to CT depends on its concentration in the feed consumed and its astringency (Mangan 1988). CT concentration may vary in response to soil fertility (Barry & Forss 1983), climate (Carter et al. 1999) or stage of plant development (Bell et al. 1992) within plant species, thereby altering the responses of grazing animals. Comparative assessment of cultivars should continue over a number of years to enable sampling over a range of environmental conditions (Morley 1978).

2.5.5 Determination of plant CT concentration

Careful consideration must be given to the methods used for CT analysis (Waghorn et al. 1999). There are numerous methods available, most of which have weaknesses associated with the heterogeneity of CT structure. The butanol-HCl (Porter et al. 1986) and vanillin-HCl (Price et al. 1978) assays are the most specific colorimetric assays used to determine unbound CT concentrations. However, the vanillin-HCl assay reacts with some phenolic compounds other than CT. Wen et al. (2003) recently quantified the concentration of unbound CT in birdsfoot trefoil using Near Infrared Reflectance Spectroscopy (NIRS) after calibration with reference data analysed by the vanillin-HCl assay. NIRS techniques can reduce the time and cost of analysis but calibrations need to be specific to the forage being analysed.

Most CT quantification methods measure only the free (unbound) portion of the CT in plants. However, harvesting, drying and grinding plant samples before analysis will rupture some vacuoles, allowing a portion of the CT to bind with plant protein or fibre. CT may also be naturally deposited in cell walls by the plant. Terrill et al. (1992a) modified the butanol-HCl procedure to achieve a repeatable extraction and determination of unbound, protein bound and fibre bound CT.

A major requirement in the quantification of CT is the use of appropriate standards that show a similar response per unit mass to the CT being measured (Scalbert 1992). Many assays respond differently to contrasting types of CT (Wisdom et al. 1987; Nelson et al. 1997; Hedqvist et al. 2000). Standards should therefore be made by purifying CT from the plant species of interest (Mole et al. 1989). This becomes

challenging for forages with very low CT concentrations, requiring vast quantities of forage to extract sufficient amounts. In such cases, it is preferable to determine the CT concentration relative to a single standard (Hagerman & Butler 1989).

The part of the plant sampled for analysis should represent that eaten by animals. Animal species vary in their grazing selection, but sheep usually select leaves over stems, which may have different CT concentrations (Waghorn et al. 1999).

2.5.6 Animal performance

Genetic improvement of pasture plants has been associated with yield, seasonality, chemical composition or disease resistance (Woodfield & Easton 2004). Such perceived gains have not always resulted in increased animal productivity or whole farm profitability (Woodward et al. 2003). Feeding value can not be predicted from chemical composition and attempts to define differences between CT from various plant species using *in vitro* procedures (Tanner et al. 1994) have not always corresponded with *in vivo* measurements (Waghorn & Shelton 1997).

Feeding trials are an essential component of forage evaluation, but responses can be affected by characteristics of individual animals (Corbett 1978), variation between animals in herbage intake (Ulyatt 1970b) and efficiency of feed utilisation by individuals (Arthur et al. 2004). Animals may be assigned to treatments by complete randomisation or by stratified randomisation (Robards 1980). Stratified randomisation will balance treatments for factors that may affect animal performance such as age, liveweight and previous production. The number of animals required to detect significant treatment effects can be determined from the variation between individuals in the parameters being measured (Corbett 1978). Randomisation and adequate animal numbers is particularly important when evaluating treatment effects on bloat, because of the large variation in susceptibility of individual animals to this disorder (Johns 1954).

There is usually a lag of 24 to 48 hours before an animal starts bloating after the commencement of feeding on bloat-provoking herbage (Reid et al. 1975), depending on the previous diet. Experiments measuring bloat should therefore cover a period greater than 48 hours. As the pressure in the rumen from bloat increases the animal is likely to stop eating, its production may be reduced, and in severe cases it may die (McClymont

1973). It is important to measure both the incidence and severity of bloat, as mild cases may be of no consequence to animal performance. The severity of bloat can be scored by a combination of visual and physical evaluation of rumen distension and rumen pressure, respectively (Johns 1954; Appendix 4.3).

Measurements of the production and persistence of rumen foam are useful for quantifying subclinical bloat, when no external signs of bloat are apparent. Bloat occurs when ruminal gases become trapped in stable and persistent foam. Good correlations have been reported between the formation of foams *in vitro* from leaf extracts and the incidence of bloat on pastures of the same plant species (Pressey et al. 1963; Kendall 1964; Cooper et al. 1966; Jones et al. 1970).

CT effects on ruminant production depend on their concentration, which may vary in response to environmental cues. Animal responses will also vary with changes in animal nutritional requirements, physiological status and diet composition (Raymond 1969). Repeating experiments over time is necessary to determine the range of likely responses.

Many studies have evaluated dietary CT by comparing forages containing CT to forages of similar quality that do not contain CT (John & Lancashire 1981; Purchas & Keogh 1984; Marten et al. 1987; Douglas et al. 1995). It has been assumed that the differences in animal responses are due to the effects of CT, however, other nutritional factors have affected the response. Administering polyethylene glycol (PEG) to animals is a useful tool for determining the effects of CT contained in their diet. PEG preferentially binds to CT, preventing CT from binding to protein (Jones & Mangan 1977), effectively inactivating the CT. The CT-PEG complex is stable over a pH range of 2 to 8.5 (Jones 1965). PEG may also disrupt already formed CT-protein complexes, as its affinity for CT exceeds that of protein (Silanikove et al. 1996). PEG has been widely used in the evaluation of CT impacts upon animal performance and digestive physiology (Wang et al. 1996a; Waghorn & Shelton 1997; Douglas et al. 1999; Woodward et al. 1999).

2.5.7 *In vitro* incubations

Animal feeding trials are the ultimate test of forage characteristics, but they are expensive, time consuming and there are often uncertainties associated with intakes and

diet composition from mixed forages. Mixtures of forage species form the basis of pastoral agriculture but the proportions of species and plant components (leaf, stem, flower) change over time. This makes it difficult to undertake grazing studies to quantify the effects of specified dietary mixes. *In vitro* incubation of plant material can overcome some of these difficulties and provides a rapid, low cost technique for feed evaluation prior to a final analysis using animal trials.

Several *in vitro* systems have been developed (Tilley & Terry 1963; Hoover et al. 1976; Broderick 1987; Waghorn & Caradus 1994) with varying degrees of complexity, and the ability to measure different aspects of digestion. The value of different methods will depend on the objectives of the research, but all simulate the processes that occur in the rumen and are subject to variables that may influence the results. It is essential that all systems maintain a normal microbial population, normal digestion rates (Warner 1956), and forage preparations should resemble grazed and chewed material (Barrell et al. 2000).

It is important that the microbial population in the *in vitro* system represents that in the rumen of animals fed a similar diet to that being tested. The use of more than one donor animal will minimise variation due to differences between individual rumen environments (Johnson 1969). Microbial populations are most active 1 to 6 hours post feeding, and the inoculum should contain some particulate material to ensure inclusion of fibre degrading bacteria. It is important that the inoculum is maintained anaerobic, and this is assisted by the use of cysteine sulphide as a reducing agent to reduce lag effects and maximise fermentation and degradation rates (Grant & Mertens 1992).

Large changes in rumen pH due to VFA production are buffered by salivation and absorption in the animal. *In vitro* incubations require careful buffering, as products of fermentation accumulate in the medium. McDougall's buffer (McDougall 1948) is based on the composition of sheep saliva and has been used with a reducing agent in *in vitro* evaluation of fresh forages (Burke et al. 2000; Chaves et al. 2002). *In vitro* incubations enable mixtures of forages containing CT to be incubated with forages which do not contain CT, in a range of proportions. These incubations are able to demonstrate probable *in vivo* responses to changing the CT content in diets grazed by

ruminants to indicate the efficiency of types or concentrations of CT on rate, extent and products of degradation.

2.6 CONCLUSIONS

New Zealand dairy pastures are characterised by high concentrations of crude protein in spring and autumn, and lack the readily fermentable energy required for microbes to capture all of the ammonia produced from ruminal protein degradation and convert it into microbial protein (Burke et al. 2002). Detoxification and excretion of excess ammonia uses energy that could otherwise have been used for milk production, and the high soluble protein concentrations in the rumen can also lead to bloat. In contrast, summer pastures may have insufficient crude protein to maximise milk production.

The potential of CT to prevent bloat and reduce ruminal protein degradation suggests their incorporation into the diet of New Zealand dairy cows will improve performance. CT in birdsfoot trefoil have consistently shown an advantage to milk production when fed to cows in mid to late lactation (Woodward et al. 1999, 2000, 2004). However, the difficulty of managing and maintaining swards containing birdsfoot trefoil or other legumes containing CT in fertile soils has limited their adoption on farm.

HT white clover was bred to increase the CT in white clover through increased flowering density and duration and increased floral CT concentrations. HT white clover presents an 'easy to use' option to supply dairy cows with CT, as white clover is a standard component of most New Zealand dairy pastures, and is of high nutritive value. The ability of HT white clover to provide a consistent supply of CT to grazing ruminants depends on the seasonality and intensity of flowering and the concentration of CT within its flowers, which may be affected by genetics and the environment. Increased flowering may, however, reduce the vegetative persistence of white clover, and therefore the CT content and feeding value of pastures.

The effects of CT on animal performance depend on dietary CT concentration and type, varying within and between plant species. The response of animals to HT white clover is therefore difficult to predict, and it requires testing concurrently with a standard cultivar under the conditions in which it must perform. Although white clover is

normally grown in mixed swards, testing in monoculture avoids complications due to varying percentages of different species in different treatments (Minson et al. 1960). Such evaluations require detailed measurements to understand observed levels of animal production and reasons for variation between and within years, including determination of the agronomic performance of the plant. Any benefits of the new cultivar in monoculture should be supported by evaluation of performance in forage mixes, thus providing a sound basis for recommendation of its use commercially, or for providing information for further development requirements of the cultivar.

CHAPTER 3

Agronomic evaluation of white clover selected for high floral condensed tannin

CHAPTER 3: Agronomic evaluation of white clover selected for high floral condensed tannin

3.1 ABSTRACT

The agronomic performance of an experimental white clover (HT) selection bred for increased condensed tannin (CT) concentrations in its flower heads and an extended flowering season was assessed under dairy cow grazing in the Waikato region of New Zealand. Three ha of HT clover and 3 ha of Grasslands Huia white clover were sown as monocultures in April 2001 at 5 kg/ha in a randomised block design, with 3 replicates, and their performance was assessed until November 2003. Stolon growing point densities were similar between treatments 7 months after sowing, but for the remainder of the experiment, were higher in Huia than HT. Huia pastures also grew approximately 500 kg/ha more clover dry matter (DM) than HT in each spring and summer. Annual DM yields averaged 11.0 and 10.0 t DM/ha for the respective clovers. Huia and HT flower heads had similar CT concentrations, but concentrations varied considerably across sampling dates. HT pastures had higher flower densities than Huia from October 2001 until January 2003, resulting in higher clover CT concentrations, but densities were similar for the remainder of the experiment. Clover CT concentrations were low (maximum of 12.1 g/kg DM) compared to legumes containing foliar tannins shown to be beneficial to ruminant production (minimum of 20 g/kg DM). Morphological examination of individual plants showed that high flower densities in HT clover were due to a higher proportion of nodes producing flowers than in Huia, but this did not significantly decrease the proportion of nodes that produced branches. Despite HT clover having similar nodal branching to Huia it was less persistent than Huia. HT pastures contained more weeds than Huia pastures and over time became contaminated with other white clover genotypes, reducing treatment differences.

This chapter forms the basis of a paper published in: *Proceedings of the New Zealand Grassland Association 65*: 139-145 (Appendix 3.1).

3.2 INTRODUCTION

White clover (*Trifolium repens* L.) is the dominant legume grown in temperate pastures, providing benefits because of its high nutritive value and ability to fix atmospheric nitrogen, and its seasonal complementarity with grasses (Caradus et al. 1996). However, problems exist with the propensity of white clover to cause bloat in ruminants and there is a large loss of protein due to its rapid degradation in the rumen (Ayres & Poppi 1993). These problems may be reduced by increasing the condensed tannin (CT) concentration in white clover. CT are secondary plant metabolites which bind to plant protein, protecting it from ruminal microbial degradation resulting in an increased amino acid flow to the small intestine (Waghorn et al. 1987a), often benefiting animal productivity.

More than 25 years of international research has failed to produce white clover containing foliar CT (Woodfield et al. 1998) although CT is present in its flower heads (Jones et al. 1976). A white clover selection (HT) recently bred for increased flower head CT concentration, a longer flowering period and a higher density of flowers (D. R. Woodfield, pers. comm.) has the potential to improve animal performance. However, CT production comes at a cost to the plant, due to competition with its primary metabolism for energy and substrates (Chew & Rodman 1979). Legumes containing CT currently used in New Zealand, such as sulla (*Hedysarum coronarium* L.) and birdsfoot trefoil (*Lotus corniculatus* L.), display inferior agronomic performance in competition with other species and are susceptible to overgrazing (Waghorn et al. 1998).

Having flower heads as the major source of CT places further limitations on the productivity and persistence of white clover. In temperate regions, white clover persists primarily by the production of horizontally spreading stolons (Harper 1978). Individual stolons have short life-spans (Chapman 1983), so persistence of the population is best achieved by dense stolon branching (Williams 1987b). However, there is an inverse relationship between stolon branching and profuseness of flowering. Each node on a stolon has the capacity to produce either a stolon branch or a flower, but not both (Williams 1987b), hence an increase in clover flower density may reduce stolon branching and plant persistence.

This chapter reports on the agronomic performance of HT and Grasslands Huia white clover, each grown in monoculture (Plate 3.1). Clover stolon density, seasonal dry matter production, flower density and CT concentrations are reported. The morphology of the two white clover lines in relation to flower and stolon production is also investigated.

3.3 MATERIALS AND METHODS

3.3.1 Plant material

Grasslands Huia (Huia) white clover and a selection of white clover (HT) bred for extended flowering and increased flower head CT concentration were used in this research. Huia was derived from New Zealand wild white No. 1 strains from the Hawke's Bay and North Canterbury regions, and was selected for yield and persistence under grazing. Huia is a medium-leaved cultivar with a dense growth habit and high phenotypic plasticity (Caradus & Woodfield 1997). Development of the HT selection is described in Section 2.4.4. The line used in this research was the F5 generation shown in Figure 2.5. HT was produced by selection for increased flower head CT concentration within Huia lines, followed by crossing with Siral white clover (an Algerian ecotype developed in Australia) for prolonged flowering.

For definitions of plant parts other than those used for clover morphology refer to Section 2.2.2 and Figure 2.1.

3.3.2 Experimental design and pasture management

The experiment was conducted at the Dexcel Lye Farm, Hamilton, New Zealand (latitude 37° 47' south, longitude 175° 19' east, altitude 40 m above sea level). The soil was a Te Rapa humic silt loam; a Humose Groundwater-Gley Podzol in the New Zealand Classification (Hewitt 1998) or a Humose Aquic Haplorthod in Soil Taxonomy (Soil Survey Staff 1990).

The experiment was a randomised block design, with three replicates. Each 2 hectare (ha) block was divided into two HT white clover paddocks and two Huia white clover paddocks of equal size (Appendix 3.2). Existing dairy pastures were killed with

glyphosate (1.44 kg a.i./ha) and dicamba (0.6 kg a.i./ha) herbicides. Paddocks were ploughed, then cultivated and fertilised with superphosphate (90 and 110 kg/ha of P and S, respectively, for replicate 1, and 59 and 72 kg/ha of P and S, respectively, for replicates 2 and 3), on 21 November 2000, bringing the soil Olsen P concentration of all replicates up to 35 mg/mL. Clover seed was roller-drilled at 5 kg/ha on 27 November, but seeds did not germinate. Paddocks were left fallow until April 2001, and then were re-sprayed with glyphosate herbicide (1.44 kg a.i./ha) and re-cultivated. Clover was sown at 5 kg/ha on 26 April 2001 by roller-drilling. Huia was Superstrike® (molybdenum, lime, nematicide and rhizobia) coated, but HT was inadvertently sown as bare seed. "Nodulaid" white clover inoculant was mixed with the seed of both clover types before sowing.

Preside herbicide (52 g/ha flumetsulam (active ingredient) plus 1 L/ha of surfactant) was applied on 19 June 2001 to control broad-leaf weed seedlings. Grasses were controlled by application of Gallant NF herbicide on 6 September 2001 (100 g/ha haloxyfop (active ingredient) + 1 L/ha of surfactant in 250 L water), 2 August 2002 (150 g/ha haloxyfop + 1 L/ha of surfactant in 250 L water) and 16 October 2003 (150 g/ha haloxyfop + 1 L/ha of surfactant in 250 L water). Asulox (1500 g/ha asulam (active ingredient)) was applied on 1 May 2002 and 19 December 2002 to control broad-leaved dock (*Rumex obtusifolius* L.). Jaguar herbicide was applied on 23 August 2002 (37.5 g/ha diflufenican and 375 g/ha bromoxynil) to control weeds, including chickweed (*Stellaria media* L.). On 4 March 2003, 5 L/ha of 2,4-DB was applied (400 g/L 2,4-DB as the sodium salt), then again on 26 June 2003 at 4.5 L/ha to control broad-leaf weeds.

MagPhos fertiliser (57, 68, 35 and 130 kg/ha of P, S, Mg and Ca, respectively) was applied to all paddocks in March 2002, as was urea (37 kg N/ha) in September 2002 and muriate of potash (50 kg K/ha) in January 2003. In April 2003, 45, 15, 55 and 101 kg/ha of P, K, S and Ca, respectively, was applied to all paddocks as potassic sulphur super and super 10, as was muriate of potash (60 kg/ha) on 29 October 2003.

Pastures were grazed by dairy cows or cut for silage when yields reached 2 to 2.5 t DM/ha, or less frequently when feed was being conserved for the grazing experiments reported in Chapter 4. Attempts at grazing on 26 September 2001 and 17 September

2003 resulted in bloat on both treatments, so cows were removed within one hour of the start of grazing; the paddocks were then cut for silage. Silage was cut on 18 October 2001, 3 January 2002, 8 January 2003 and 4 October 2003. Complete grazing of all paddocks occurred in November/December 2001, January/February 2002, March/April 2002, June 2002, November/December 2002, March/April 2003 and May/June 2003.

3.3.3 Seed weight, purity and germination

A total of one thousand Huia white clover seeds from the seed line used in this experiment were weighed on a Sartorius balance (Model BA210S, accurate to 0.0001 g) in lots of 100 in August 2001. Similar information was obtained for the HT line used (D. R. Woodfield, unpublished data). Information on seed purity and germination percent was obtained from the seed analysis certificate on the Huia seed line (National Seed Laboratory, New Zealand), and from tests on the HT seed line (D. R. Woodfield, unpublished data).

Five and eleven weeks after sowing, clover seedlings were counted in 60 randomly located 0.12 m^2 quadrats per paddock to estimate seedling establishment density of the two clovers.

3.3.4 Herbage dry matter accumulation

Twelve 0.5 m^2 grazing exclosure cages (Plate 3.2) were placed in each treatment (one in the front and back half of each paddock) in early December 2001, and the herbage within was trimmed to a height of 2 cm using an electric shearing handpiece. After 3 to 8 weeks regrowth (with cutting interval depending on growth rate), herbage mass was estimated by cutting a 0.2 m^2 quadrat to a 2 cm stubble within each cage. The cage was then relocated and the herbage within was trimmed to a 2 cm stubble. This procedure was repeated until December 2003. Cages were rotated clockwise within each paddock half by moving the cage approximately 10 m after each cut, avoiding fence lines and troughs, and areas containing weeds. The cut herbage was dried for 36 hours at 95°C in a forced-draught oven before weighing. Seasonal and annual herbage production was calculated from these data.

3.3.5 Clover growing point, flower and seedling densities

From December 2001 to July 2002, clover density was assessed bi-monthly in fifteen randomly located tiller frames (5 x 20 cm) per paddock; assessments were monthly from July 2002 to November 2003. The number of clover growing points, clover flowers and clover seedlings were counted in each quadrat. Measurements were made within the week before grazing or cutting of the clover pastures, allowing maximum expression of flowering.

3.3.6 Clover stolon dry weight

Stolon dry weight per m^2 was measured every 3 months from July 2002 to October 2003. Three turves were removed from each paddock (front, middle and back) from random locations by digging around the edges of a 30 x 30 cm quadrat with a spade to a depth of 5 cm. Clover plants were thoroughly washed to remove soil. All root, leaf and flower material were cut from the stolons at their point of attachment and discarded. Stolon samples were oven dried at 95°C for 48 hours, then weighed.

3.3.7 Clover morphology

One turf was removed from each paddock bi-monthly from November 2001 to November 2003. The location of the turf was rotated between the front, middle and back third of each paddock, avoiding fence lines, troughs, and areas containing weeds. If measurements coincided with grazing or silage cutting, turves were collected in the week before defoliation, to allow maximum expression of morphological characters. Turves were removed by cutting around the edges of a 62 x 62 cm quadrat with a spade to approximately 5 cm depth. Soil was washed from each turf, and all clover plants were separated intact. Plants with stolons cut at the quadrat edge were discarded. The number of uncut tap rooted and clonal plants per turf were recorded.

Twenty intact plants were randomly selected from each turf for morphology measurements. For every stolon on each of the 20 plants, node, leaf, branch and flower numbers were counted; presence or absence of a growing point was noted and stolon length was measured to the nearest mm. Plants were assigned an order according to the level of stolon branching, such that plants with a single stolon were first order, plants with one level of branching off the parent stolon were second order, plants with one

level of branching off secondary stolons were third order, and so on. The following definitions were used for clover morphological characters:

- Leaf: All leaves with leaflets separated and 50% or more unfolded (growing points were excluded).
- Flower: Any visible flower, including immature buds.
- Branch: Vegetative shoot from a leaf axil with a visible stolon, supporting at least two leaves (Thomas 1987).
- Stolon length: Measured from branch base to the base of the youngest open leaf.

In previous experiments (Brock et al. 1988; Harris 1994) where small (625 to 900 cm^2) turves were removed for clover morphology measurements, data were weighted to account for bias against larger plants intersecting the quadrat boundaries. Nevertheless, Harris (1994) found a quadrat size of 900 cm^2 in dairy pastures produced similar results for weighted and unweighted data, thus providing an unbiased sample of the clover plants present. The larger quadrat size used in this experiment (3844 cm^2) would have reduced the likelihood of a biasing effect even further, therefore data were not weighted in this experiment.

3.3.8 Condensed tannins and botanical composition

CT concentration was measured bi-monthly on white clover flower heads from November 2001 to May 2002 and then monthly (when flower heads were present) for the remainder of the experiment. A herbage sample from each paddock was cut with hand shears to 1 to 2 cm above ground level (so as to avoid cutting stolons), generally before grazing. Each sample was mixed thoroughly before dissecting into clover flower heads, the remainder of the clover plant (peduncles and leaves; Figure 2.1), and other plant species.

A 50 g sample of fresh clover flower heads from each paddock was frozen and stored at -18°C before freeze-drying, grinding through a 1 mm sieve and analysed using the butanol-HCl colorimetric technique (Terrill et al. 1992a) to determine free, protein bound, and fibre bound CT concentrations for the November 2001 to May 2002 samples. Due to the difficulty of obtaining enough white clover flowers, lotus major

(*Lotus pedunculatus* Cav.) CT was used as a standard for the analyses of CT. Samples were then scanned to establish calibration curves for future CT analysis by near infrared spectroscopy (NIRS; Corson et al. 1999). Calibration statistics are presented in Appendix 3.3. CT concentrations from samples collected from October 2002 to November 2003 were determined by NIRS to reduce analytical costs.

Grazing animals ingest not only clover flower heads, but also peduncles and leaves, which do not contain CT. To estimate the concentration of CT in the clover herbage (clover CT), the flower head CT concentration of each sample was multiplied by the proportion of flower heads in the DM of the clover collected in each sample.

To estimate the botanical composition of the pasture on a dry matter basis, the fresh weight of dissected clover flower heads, the remainder of the clover plant and other pasture components were recorded, and a sub-sample of each was weighed, dried at 95°C for 36 hours, then weighed again to determine DM%. For components weighing less than 300 g the whole sample was dried and weighed.

3.3.9 Cyanogenesis

In 2003, clover pastures had patches of clover that were morphologically atypical of the rest of the pasture. Some plants had red (anthocyanin) leaf markings, others had prominent white leaf markings, and still others had larger, lighter grey-green coloured leaves with less pronounced leaf markings. This indicates that the pastures may have been contaminated with clover other than that of the variety that was sown.

Cyanogenic plants account for 80% of Huia (Caradus & Woodfield 1997) and 100% of HT white clover plants (D. R. Woodfield, unpublished data). If paddocks sown with HT clover have less than 100% of clover leaves testing positive for cyanogenesis this would be a further indication that they were contaminated with volunteer (non-sown) white clover.

Twenty leaves were collected from each paddock in January 2004. Samples were collected randomly, with 10 paces between each sample, so as to avoid collecting more than one leaf from the same plant. Each leaf was tested for cyanogenesis (Appendix 3.4) using a modification of the Guignard picrate paper test (Corkill 1940).

3.3.10 Statistical analysis

Data were subjected to analysis of variance using Genstat 5, version 4.2 (Genstat 5 Committee 1997) for detecting differences between treatments at individual sampling dates. Flower and seedling density data were analysed using the raw data, but as some data were skewed, a $\log_{10}$ transformation was performed and the data were re-analysed. As the conclusions were similar for raw and transformed flower density data, only the raw data are presented for ease of interpretation. On some dates no flowers or seedlings were present in one or both treatments, so data could not be analysed.

For flower head CT concentration analysis, the dates where bulk samples were collected across treatments were omitted from analyses. The effect of sampling date on condensed tannin concentrations was analysed by repeated measures analysis using the AREPMEASURES procedure in Genstat 7.1 (Genstat Committee 2003). This procedure adjusts the degrees of freedom for time and interactions with time using Greenhouse-Geisser corrections. Concentrations of free, bound and total flower head CT were analysed after square root transformation, and percentages of free and bound CT were analysed after angular transformation. As conclusions were similar for transformed and non-transformed data, the non-transformed data are presented for ease of interpretation.

PLATE 3.1 Boundary between Huia (left) and HT (right) white clover paddocks in November 2001.

PLATE 3.2 Grazing exclosure cage used for the measurement of herbage accumulation.

3.4 RESULTS

3.4.1 Climate

Weather records during the experiment are given in Appendix 3.5. These data were collected at the Ruakura Climatological Station, 4 km from the experimental site. The Waikato region has approximately 1200 mm rainfall per annum with an even distribution, but tending towards summer dry. Monthly grass minimum temperatures average 1°C in winter (June, July, August) and 9°C in summer (December, January February), with average monthly maximum air temperatures of 14 and 23°C, respectively. Conditions in autumn 2001 were good for establishment, with sufficient rainfall and warmer than average temperatures. July 2001 was colder than average, with 17 frosts, but was followed by a warm spring (September to November). The first summer (December 2001 to February 2002) had only 66% of the average rainfall (206 mm). The start of the second summer (December 2002, January 2003) was wetter than normal, but February 2003 had no rain in the first two weeks, and only 9 mm in the third week.

3.4.2 Seed weight and germinability

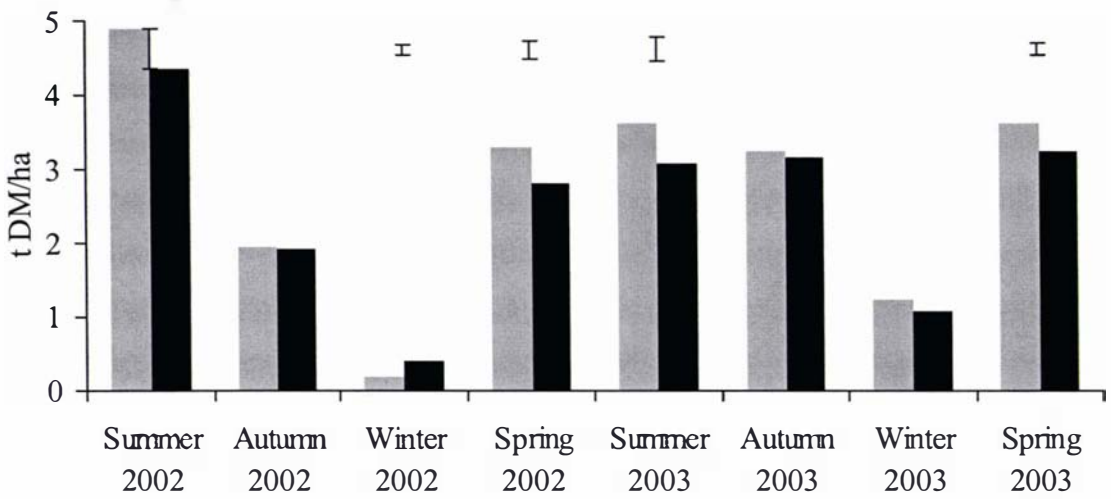
The average thousand seed weight of Superstrike® coated Huia seeds was 1.13 g, and that for uncoated HT seed was 0.75 g. The percentage of germinable seed was 92% for Huia and 96% for HT. The purity of the Huia and HT seed lines was 99.2% and 99.9%, respectively. This indicates that HT white clover seed was sown at a higher seed rate than Huia (639 versus 404 germinable white clover seeds/m^2).

Clover seedling densities at establishment were greater for HT than Huia, both five weeks (352 versus 204 seedlings/m^2; $P < 0.05$) and eleven weeks (313 versus 187 seedlings/m^2; $P < 0.01$) after sowing (26 April 2001).

3.4.3 Herbage accumulation

HT clover grew approximately 500 kg DM/ha less herbage over spring (September to November) and summer (December to February) than Huia (Figure 3.1). Autumn (March to May) herbage accumulation was similar for both treatments, and was higher for HT than Huia in the first winter (June to August 2002), but similar in the second winter (June to August 2003).

FIGURE 3.1 Seasonal accumulation of white clover herbage in pastures of Huia (■) or HT (■) white clover from summer 2002 to spring 2003. Error bars represent LSD ($P < 0.05$).



Total clover herbage accumulation in Year 1 (December 2001 to December 2002) was lower for HT (9486 kg DM/ha) than for Huia (10331 kg DM/ha) ($P < 0.05$). Comparable data for Year 2 were 10562 and 11710 kg DM/ha ($P = 0.067$). Total herbage accumulation over the 2 years was 2.0 tonne greater for Huia than HT ($P < 0.05$).

3.4.4 Botanical composition

Pastures were sown as pure clover and herbicides were used to control other species. Following establishment, there was a low weed content until autumn 2002. Herbage dissections in May 2002 and July 2002 showed a lower proportion of clover in the HT treatment than in Huia, with the remainder of the pasture being annual poa (*Poa annua* L.) and chickweed (*Stellaria media* L.). Clover contents were lowest in July 2002, representing 84 and 68% of DM in Huia and HT, respectively ($P < 0.05$). Clover content was higher in Huia than HT for the remainder of the experiment, although differences were usually non-significant.

The proportion of harvestable clover (leaf plus flower) DM as flower heads was consistently highest in HT, peaking in March 2002 and again in January 2003 (Table

3.1). Flower heads made up at least twice as much of the clover DM in HT compared to Huia until January 2003. For the remainder of the experiment differences between treatments were small and not always significant.

TABLE 3.1 Percentage of pasture DM as white clover, and percentage of white clover DM as flower heads in Huia and HT white clover pastures.

Date	% of DM as white clover			% of clover DM as flower heads		
	Huia	HT	SED	Huia	HT	SED
November 2001	100.0	100.0	0.00^{NS}	3.6	9.9	0.69*
January 2002	100.0	100.0	0.00^{NS}	4.9	15.3	0.59**
March 2002	100.0	100.0	0.00^{NS}	13.2	21.2	1.35*
May 2002	88.2	77.2	3.10**	1.2	2.9	0.42^{NS}
July 2002	84.0	67.5	6.79*	0.0	0.1	NA
September 2002	98.8	95.7	3.47^{NS}	0.0	0.5	NA
October 2002	98.2	92.5	3.52^{NS}	0.0	0.7	0.01**
November 2002	97.5	87.5	2.28**	3.2	10.0	0.27**
December 2002	100.0	97.8	1.99^{NS}	4.7	14.1	2.01*
January 2003	90.0	89.2	1.92^{NS}	12.8	22.0	0.96*
February 2003	94.2	86.7	4.11^{NS}	12.6	14.2	0.45^{NS}
March 2003	93.7	84.2	3.61*	11.5	14.4	0.95^{NS}
April 2003	96.1	91.5	2.62^{NS}	5.2	7.7	0.45*
May 2003	90.8	88.2	2.62^{NS}	0.7	1.4	0.04**
July 2003	95.7	81.5	4.18†	0.0	0.0	NA
September 2003	84.2	77.2	6.51^{NS}	0.1	0.3	0.11†
October 2003	84.6	79.3	4.22^{NS}	0.5	2.6	0.68†
November 2003	94.0	87.0	3.69^{NS}	6.6	10.9	1.89^{NS}

^{NS} = non significant; † = $P < 0.1$; * = $P < 0.05$; ** = $P < 0.01$ for the significance of differences between treatment means within dates.

NA = not analysed, statistical analysis was not possible because of too many zeros.

3.4.5 Clover stolon growing point, flower and seedling density

Clover growing point density in November 2001 was similar between treatments (average of 873/m^2). From January 2002 onwards densities were consistently greater in Huia than HT (Figure 3.2) although differences were not always significant, especially in the last seven months of the experiment. Both treatments followed the same seasonal pattern of growing point densities, with peaks in September 2002 (Huia 2724, HT 1680/m^2; $P < 0.05$) and August 2003 (Huia 3210, HT 2427/m^2; $P = 0.08$).

The density of clover flowers was consistently greater in HT (Figure 3.3). Flower buds were present throughout the year in HT, but did not appear in Huia until September/October. Fully developed flowers were first evident in September for Huia and HT in 2001 and 2003, but in 2002 they did not appear until early October for HT, and late October for Huia. Clover flower densities showed peaks in November/December and March each year. Peak flower densities in HT were at least double those of Huia in November 2001, March 2002 and December 2002, but for the remainder of the experiment (February 2003 to November 2003), differences between treatments were small.

The number of clover flowers per stolon growing point (Figure 3.4) followed similar trends to that of flower density, with HT having more flowers per growing point than Huia until February 2003, when treatment differences became small.

Following establishment in April 2001, further clover seed germinated during the experiment (Table 3.2). Seedling densities were greatest in autumn (March, April, May) 2003, but treatment effects were usually non-significant.

FIGURE 3.2 Density of clover stolon growing points in pastures of Huia and HT white clover from November 2001 to November 2003. Error bars represent LSD ($P < 0.05$). Treatment differences close to significance ($P < 0.1$) are represented by †.

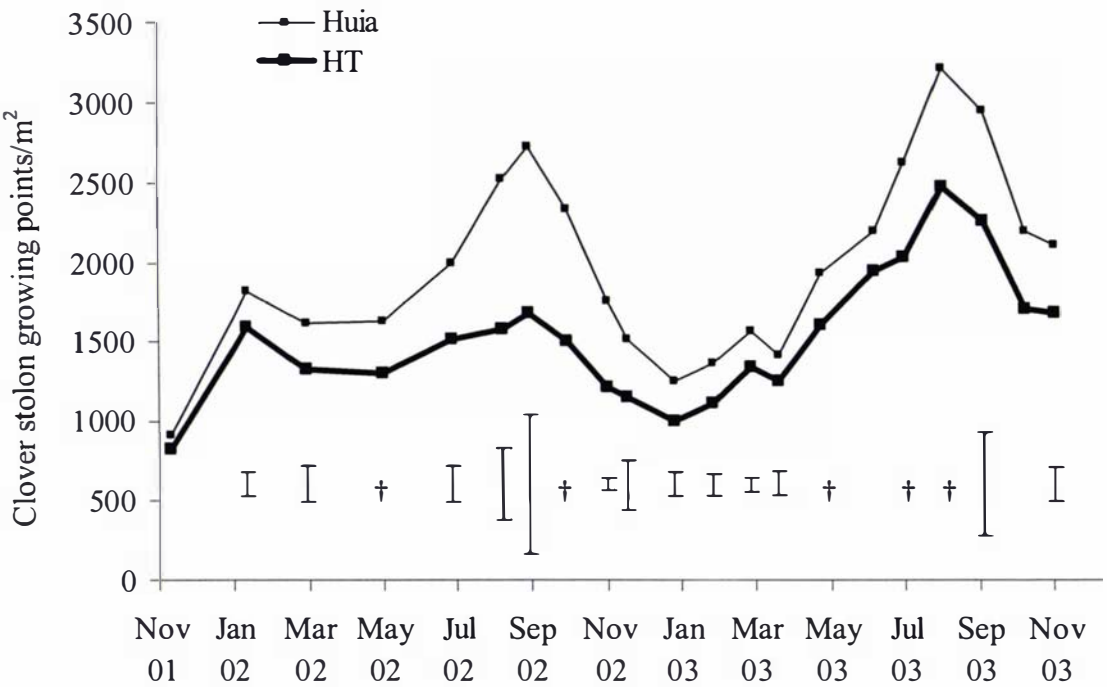


FIGURE 3.3 Density of clover flowers in pastures of Huia and HT white clover from October 2001 to November 2003. Error bars represent LSD ($P < 0.05$). Treatment differences close to significance ($P < 0.1$) are represented by †.

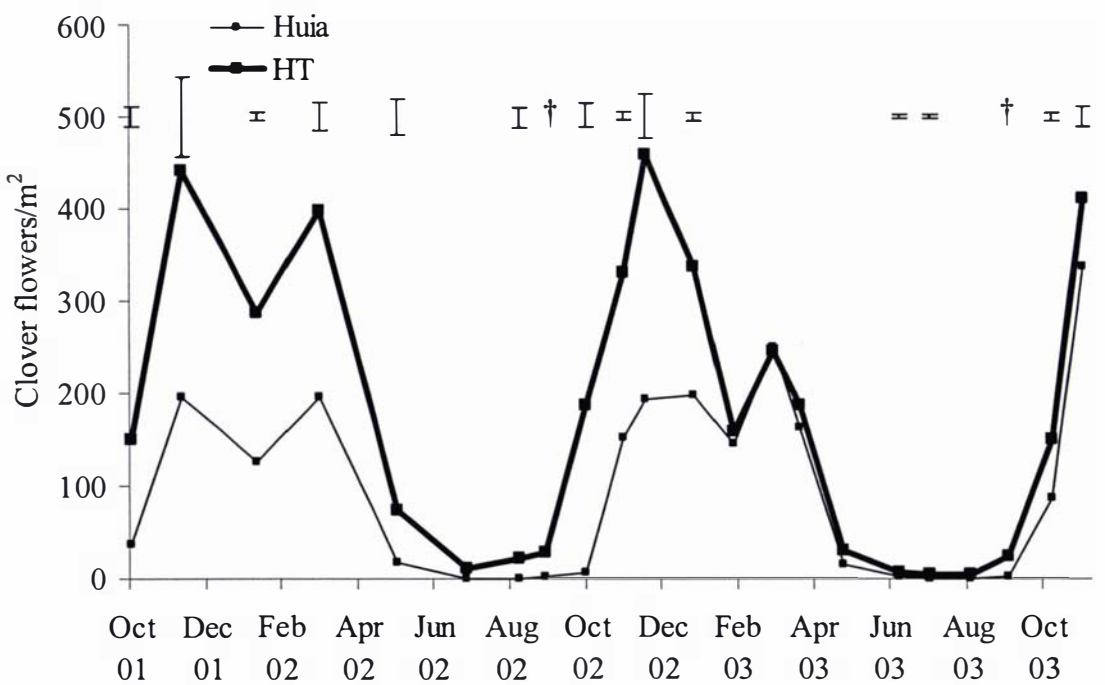


FIGURE 3.4 Average number of flowers per growing point for Huia and HT white clover pastures from November 2001 to November 2003. Error bars represent LSD ($P < 0.05$). Treatment differences close to significance ($P < 0.1$) are represented by †.

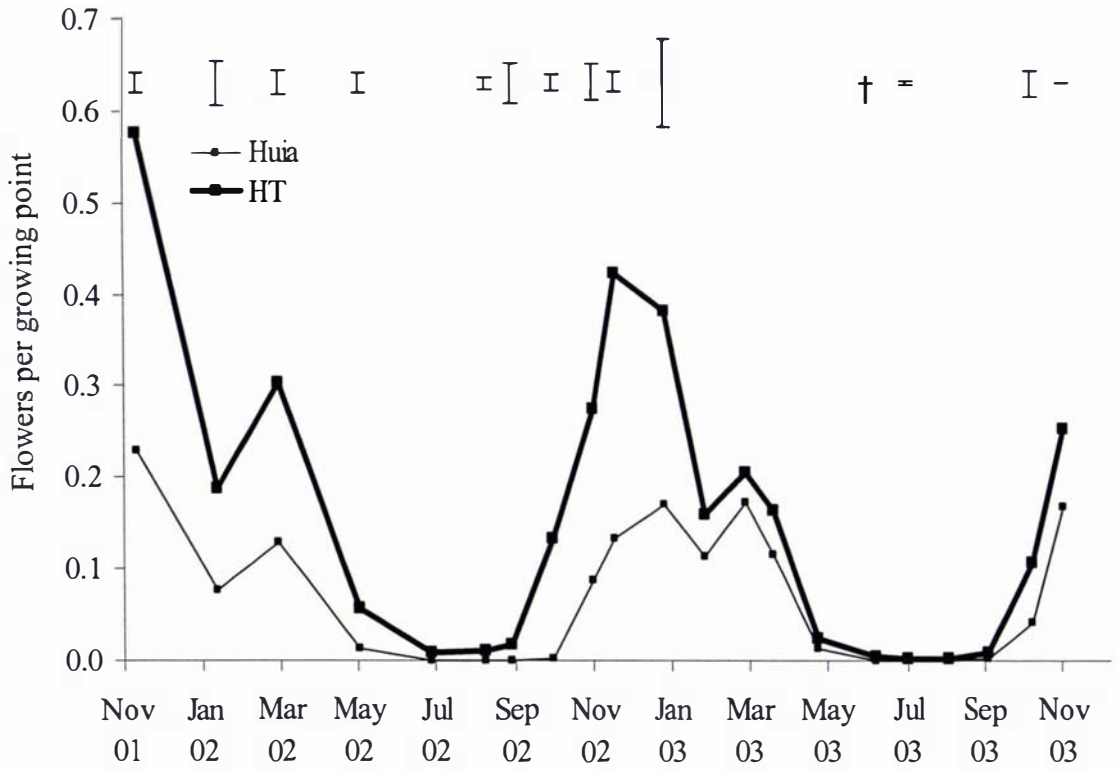


TABLE 3.2 Density of clover seedlings in Huia and HT white clover pastures from November 2001 to November 2003. Data are only presented for months when seedlings were present[1].

Month	Clover seedlings/m^2		Log_{10} transformed clover seedling density			
	Huia	HT	Huia	HT	SED	P
January 2002	9	76	0.76	1.84	0.250	0.050
March 2002	18	87	1.12	1.75	0.477	0.321
May 2002	69	203	1.67	2.11	0.429	0.413
July 2002	1	1	0.21	0.21	0.368	1.000
March 2003	90	40	1.94	1.03	0.652	0.298
April 2003	440	537	2.64	2.69	0.148	0.749
May 2003	143	223	2.08	2.35	0.181	0.277
September 2003	0	1				NA
October 2003	23	13	1.03	0.54	0.147	0.078

[1] No seedlings were observed in November 2001, August 2002 to February 2003, June 2003 to August 2003 or November 2003. NA = not analysed.

3.4.6 Stolon dry matter yield

Stolon mass (kg DM/ha) was greater in Huia than HT pastures for the first two measurements (winter 2002 and spring 2002), then was similar between treatments for the remainder of the experiment (Table 3.3). Stolon mass was low in spring, and high in summer and autumn. Winter stolon mass was low in 2002, but high in 2003.

TABLE 3.3 Stolon mass (kg DM/ha) in Huia and HT white clover pastures.

Month	Huia	HT	SED	P
Winter (July) 2002	1630	1150	101.3	0.042
Spring (October) 2002	1650	1300	28.4	0.007
Summer (January) 2003	2265	2027	201.4	0.429
Autumn (April) 2003	2163	2079	91.6	0.484
Winter (July) 2003	2015	2047	78.1	0.724
Spring (October) 2003	1576	1436	94.9	0.278

3.4.7 Clover morphology

Proportion of tap rooted plants

At the first measurement in November 2001, 7 months after sowing, most plants were still tap rooted (Table 3.4), with a larger percentage in Huia than HT (97 versus 85% of plants, $P < 0.05$). Following the first grazing in November/December 2001, there was a substantial decrease in the proportion of tap rooted plants, with a larger drop in Huia than HT pastures. From January to May 2002 there were more tap rooted plants in HT than Huia, but there were only 2% of plants tap rooted in both treatments by July 2002, with all plants in the clonal growth stage from September 2002 onwards. The faster loss of tap rooted plants in Huia than HT was reflected in the higher proportion of first order plants in Huia than HT in January and March 2002 (Figure 3.5).

TABLE 3.4 Change in the percentage of tap rooted clover plants in Huia and HT white clover pastures from November 2001 to July 2002.

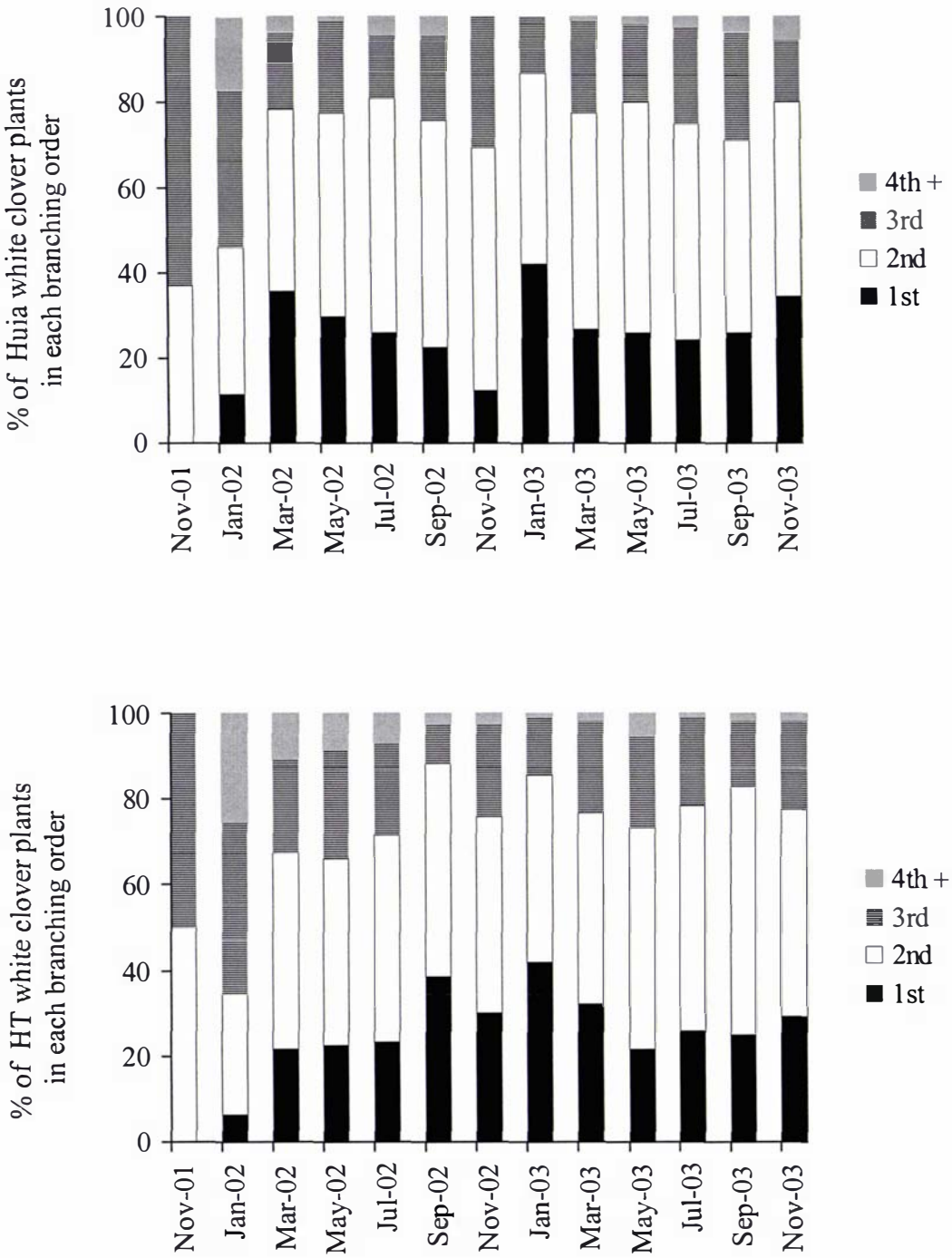
Month	Huia	HT	SED	P
November 2001	97	85	4.2	0.025
January 2002	43	64	11.3	0.094
March 2002	8	22	5.1	0.023
May 2002	2	11	4.2	0.059
July 2002	2	2	1.7	0.631

Plant branching order

The average distribution of plants amongst branching orders was 27% first order, 47% second, 21% third, 4% fourth and 1% fifth and sixth order. At the first measurement (November 2001), before paddocks had been grazed, all plants were second and third order (Figure 3.5). At the second measurement there was a small proportion of first order plants, with more in Huia than HT (12 versus 7%, respectively, $P = 0.07$), and the highest proportion of fourth and higher order plants for the duration of the experiment in both treatments (17 and 25% for Huia and HT, respectively). For the remainder of the experiment there were first to fourth order or higher plants at each measurement, except for Huia in November 2002 and January 2003, which had no fourth or higher order plants.

The percentage of first order plants in 2002 peaked in March for Huia, (36% versus 22% for HT; $P = 0.06$), then steadily declined to a low of 13% in November (compared to 30% for HT; $P = 0.07$). For HT, first order plants increased from January 2002, peaking in September 2002 (39%). HT had a higher percentage of fourth and higher order plants than Huia in March 2002 (3 versus 11%; $P < 0.05$), and a lower percentage of third order plants in September (9 versus 20%; $P < 0.05$) and November (31 versus 22%; $P < 0.01$) 2002. The distribution of plants among branching orders was similar between treatments in 2003, with the highest percentage of first order plants in January (42%).

FIGURE 3.5 Distribution of clover plants among branching orders for Huia and HT white clover pastures, where 1st represents first order plants, 2nd is second order plants, 3rd is third order plants and 4th + represents fourth, fifth and sixth order plants.



Plant morphology

Huia had more stolons and therefore more growing points and a greater total stolon length per plant than HT at the first measurement in November 2001 (Table 3.5). This was probably due to the larger percentage of tap rooted plants in Huia, indicating less fragmentation had occurred. There were no treatment effects on these parameters for the remainder of the experiment. The percentage of nodes with a stolon branch was similar between treatments for most of the experiment, except in September 2002, when Huia had 12.0% branching compared to 9.6% for HT, corresponding with the high percentage of first order plants for HT at this time. Branching was highest at the first measurement (14% of nodes) before substantial fragmentation of plants had occurred. Branching then varied throughout the year for both treatments from 2 to 12% of nodes.

The number of flowers per plant and per node was higher in HT than Huia from November 2001 to January 2003, but was similar in both treatments for the remainder of the experiment. The number of flowers per plant and per node was highest in November each year for HT, but declined from 5.6% of nodes in 2001 to 2.9% in 2002 and only 2.1% in 2003. In contrast, Huia peaked in March 2002 with 1.4% of nodes having flowers and in March 2003 with 1.6%.

Stolon morphology

The percentage of stolons with a growing point was always high, never falling below 85%, and was similar between treatments (Table 3.6). The average internode length was short (4 to 9 mm), but tended to be longer in HT than Huia, with significant differences from July 2002 to November 2002 ($P < 0.05$), and close to significance in September 2003 ($P < 0.1$). HT stolons tended to be longer than those of Huia plants during this time, as well as in May 2002, but HT had shorter stolons than Huia in July 2003.

The average number of leaves per stolon was similar between treatments, and ranged from 1.6 to 2.8, with the highest values in summer. Individual stolons had a maximum of three flowers at any one time for both Huia and HT. HT tended to have more flowers per stolon than Huia until January 2003, but treatments were similar for the rest of the experiment (Table 3.6). Huia had slightly more branches per stolon than HT in November 2001 and November 2002 ($P < 0.1$).

TABLE 3.5 Morphological characteristics of white clover plants in Huia and HT white clover pastures from November 2001 to November 2003.

Month	Treatment	Stolons /plant	Growing points /plant	Stolon length/ plant (mm)	Flowers /plant	% nodes with flowers	% nodes with branches
Nov 01	Huia	11.1	10.6	497	1.09	1.37	13.9
	HT	5.8	5.6	285	2.13	5.56	13.5
	SED	1.06*	1.17*	56.1†	0.126*	0.035**	0.51
Jan 02	Huia	9.9	9.2	468	0.68	1.38	10.2
	HT	11.6	10.3	526	1.50	2.26	11.3
	SED	2.23	0.81	125.2	0.247†	0.066**	1.16
Mar 02	Huia	3.3	3.1	166	0.37	1.41	6.0
	HT	4.7	4.0	211	1.43	3.71	7.4
	SED	0.48	0.57	38.4	0.161*	1.15	0.70
May 02	Huia	4.4	4.2	166	0.02	0.07	7.3
	HT	4.8	4.4	205	0.33	0.81	7.6
	SED	0.83	0.70	36.5	0.039*	0.085*	0.70
July 02	Huia	5.4	4.8	172	0.00	0.00	7.6
	HT	4.8	4.4	201	0.04	0.11	7.2
	SED	0.65	0.34	27.4	0.008*	0.06	0.50
Sep 02	Huia	5.7	5.2	161	0.00	0.00	12.0
	HT	3.4	3.2	174	0.07	0.11	9.6
	SED	1.42	0.94	34.9	0.008*	0.016*	0.38*
Nov 02	Huia	4.8	4.3	229	0.49†	0.99†	6.3
	HT	3.8	3.5	278	1.07	2.87	5.5
	SED	0.47	0.55	27.2	0.138	0.541	0.83
Jan 03	Huia	2.3	2.1	203	0.51	1.39	2.4
	HT	3.0	2.7	224	0.89	2.55	4.0
	SED	0.59	0.31	61.0	0.128†	0.08**	0.75
Mar 03	Huia	3.8	3.0	214	0.74	1.55	5.2
	HT	3.2	2.6	212	0.58	1.79	5.2
	SED	0.45	0.60	12.4	0.151	0.122	2.62
May 03	Huia	3.5	3.3	150	0.01	0.06	7.1
	HT	4.1	3.8	183	0.13	0.65	9.3
	SED	0.51	0.44	40.8	0.046	0.262	1.3
July 03	Huia	4.9	4.5	174	0.02	0.07	7.4
	HT	4.2	3.8	145	0.12	0.48	7.0
	SED	0.71	0.66	22.4	0.100	0.336	1.04
Sep 03	Huia	5.1	4.6	158	0.00	0.00	9.0
	HT	4.0	3.4	155	0.03	0.04	6.2
	SED	0.69	0.55	23.3	0.014	0.020	1.34
Nov 03	Huia	3.1	2.5	131	0.42	1.05†	4.9
	HT	3.2	2.6	148	0.67	2.11	6.0
	SED	0.51	0.44	31.4	0.151	0.257	0.93

† = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.01$ for differences between treatments within months.

Data with no symbol were not significantly different.

TABLE 3.6 Morphological characteristics of white clover stolons in Huia and HT white clover pastures from November 2001 to November 2003.

Date	Treatment	Stolon length (mm)	Internode length (mm)	Growing points/stolon	Flowers /stolon	Branches/ stolon
Nov 01	Huia	45	7.7	0.98	0.095	0.89
	HT	48	8.5	0.98	0.375	0.80
	SED	4.1	0.39	0.004	0.028*	0.022†
Jan 02	Huia	54	8.2	0.93	0.009	0.72
	HT	49	7.2	0.91	0.191	0.76
	SED	1.7	0.96	0.005†	0.021*	0.025
Mar 02	Huia	30	6.0	0.98	0.142	0.63
	HT	39	5.8	0.98	0.315	0.75
	SED	6.9	0.28	0.036	0.059†	0.058
May 02	Huia	46	5.8	0.94	0.007	0.50
	HT	52	5.9	0.91	0.081	0.57
	SED	1.1*	0.51	0.048	0.015*	0.029
July 02	Huia	41	4.8	1.00	0.000	0.73
	HT	49	5.3	1.00	0.018	0.74
	SED	5.6	0.08*	0.000	0.0159	0.008
Sep 02	Huia	31	3.6	0.98	0.000	0.76
	HT	64	5.2	1.00	0.012	0.60
	SED	5.2*	0.11**	0.017	0.003†	0.115
Nov 02	Huia	54	4.9	0.91	0.126	0.62
	HT	75	6.6	0.95	0.373	0.52
	SED	5.6†	0.18*	0.038	0.049*	0.025†
Jan 03	Huia	88	5.4	0.92	0.254	0.35
	HT	89	5.8	0.90	0.407	0.38
	SED	16.8	0.99	0.045	0.035*	0.066
Mar 03	Huia	70	5.3	0.87	0.303	0.51
	HT	79	6.4	0.88	0.219	0.48
	SED	3.8	0.47	0.043	0.121	0.042
May 03	Huia	47	5.7	0.94	0.004	0.51
	HT	52	6.4	0.94	0.065	0.58
	SED	7.7	0.68	0.021	0.024	0.051
July 03	Huia	45	4.4	0.94	0.008	0.59
	HT	42	4.4	0.93	0.058	0.55
	SED	0.6*	0.23	0.008	0.050	0.068
Sep 03	Huia	38	3.9	0.91	0.000	0.79
	HT	46	4.4	0.88	0.003	0.53
	SED	0.5	0.16†	0.017	0.002	0.232
Nov 03	Huia	32	5.3	0.85	0.102	0.44
	HT	35	6.6	0.86	0.135	0.48
	SED	6.1	1.19	0.028	0.019	0.060

† = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.01$ for differences between treatments within dates.
Data with no symbol were not significantly different.

Intact plants per turf

In November 2001, HT turves supported more intact plants per turf than those from Huia pastures (Table 3.7). For the remainder of the experiment, no significant treatment differences were detected, although Huia turves tended to have higher plant numbers than HT, except in July 2003. Plant numbers per turf were similar at the first and second measurement, but there was about a five-fold increase in the number of intact plants per turf by the third measurement (March 2002). The number of intact plants per turf in the first year of measurements peaked at 155 in May for HT, and at 260 for Huia in September. In 2003, both treatments peaked in November, with approximately 300 intact plants per turf. However, the number of intact plants per turf does not give an accurate estimate of treatment plant density, since the treatment with smaller plants is likely to have a larger proportion of its plants uncut, inflating the count of intact plants per turf.

TABLE 3.7 Average number of intact white clover plants from turves (62 x 62 cm) collected from Huia and HT white clover pastures.

Month	Huia	HT	SED	P
November 2001	27	36	2.09	0.044
January 2002	26	30	5.99	0.588
March 2002	138	139	21.0	0.961
May 2002	188	155	19.9	0.240
July 2002	203	104	33.7	0.099
September 2002	260	144	52.6	0.160
November 2002	127	109	39.7	0.705
January 2003	185	133	33.1	0.261
March 2003	169	146	16.4	0.303
May 2003	230	178	39.7	0.320
July 2003	237	260	29.6	0.524
September 2003	239	223	37.1	0.700
November 2003	312	277	27.0	0.323

3.4.8 Condensed tannin concentration

Total CT concentration in flower heads ranged from 13 to 80 g/kg DM and was similar between treatments. Because of a higher density of flower heads in HT clover pastures, clover CT was higher in HT pastures, although this was not always significant (Table 3.8). There were no significant ($P < 0.05$) differences between treatments in the proportion or concentration of free, protein bound and fibre bound condensed tannins in clover flower heads when data were pooled across sampling dates.

Concentrations and proportions of free, protein bound and fibre bound CT varied among sampling dates ($P < 0.001$). There were no interactions between treatment and sampling date for any CT fractions. Total flower head CT concentrations (mean of Huia and HT) varied from 15 to 77 g/kg DM, and followed the same trend over time as free CT concentrations (Table 3.9). Concentrations in September/October were intermediate to high, and were highest in November and December of each year. Values were variable in January, and intermediate to low from February to April, and intermediate in May. Free CT usually made up the greatest proportion of total CT, followed by protein bound, then fibre bound, except in April 2003, when total CT concentrations were very low.

Although there was no strong relationship between flower head CT concentrations and season, the stage of flower head development appeared to have some influence (Table 3.10). When flower heads were in full bloom the CT concentrations were high, but concentrations were low when flower heads were senescent, or when they were green with no petals. Concentrations were highest at peak flowering (November/December).

TABLE 3.8 Total condensed tannin concentration in clover flower heads, and clover plant (leaf plus flower) in Grasslands Huia and HT white clover pastures.

Month	CT in flower heads (g/kg DM)			CT in clover plant (g/kg DM)		
	Huia	HT	SED	Huia	HT	SED
November 2001	65	70	2.3^{NS}	2.3	7.0	0.98^{*}
January 2002	70	79	$2.6^{\dagger}$	3.4	12.1	0.72^{**}
March 2002	39	40	6.8^{NS}	5.0	8.7	1.39^{NS}
May 2002	45	53	5.7^{NS}	0.6	1.5	0.07^{**}
October 2002	-	46[a]		0.0	0.3	
November 2002	75	79	2.2^{NS}	2.4	7.9	0.35^{**}
December 2002	71	75	2.8^{NS}	3.3	10.7	1.53^{*}
January 2003	33	30	2.2^{NS}	4.3	6.5	0.48^{*}
February 2003	47	47	1.5^{NS}	5.7	6.5	0.31^{NS}
March 2003	28	30	2.1^{NS}	3.2	4.2	$0.27^{\dagger}$
April 2003	17	13	$1.2^{\dagger}$	0.9	1.0	0.08^{NS}
May 2003	53	54	0.2^{*}	0.4	0.8	0.07^{**}
September 2003	68[a]	80[a]		0.1	0.3	
October 2003	75[a]	60[a]		0.4	1.6	
November 2003	64	67	2.1^{NS}	4.3	7.3	1.14^{NS}

- = no flower heads were present.

[a] = One bulk sample collected across all paddocks within the same treatment.

^{NS} = non significant; † = $P < 0.1$; * = $P < 0.05$; ** = $P < 0.01$ for differences between treatments within the same month.

TABLE 3.9 Concentration (g/kg DM) and distribution of free, protein bound and fibre bound condensed tannin in flower heads of white clover (average of Huia and HT).

Month	Free CT	CP CT	Fibre CT	Total CT	% Free CT	% CP CT	% Fibre CT
Nov 01	44.8	20.0	2.97	67.7	67	29	4
Jan 02	49.8	23.0	1.93	74.7	66	31	3
Mar 02	17.0	19.5	2.71	39.2	44	49	7
May 02	21.7	24.0	3.14	48.8	44	49	7
Oct 02[1]	37.7	6.7	1.90	46.3	81	15	4
Nov 02	58.3	16.6	2.14	77.0	75	22	3
Dec 02	55.1	15.4	2.93	73.4	75	21	4
Jan 03	18.6	8.9	3.96	31.4	59	28	13
Feb 03	41.3	5.1	0.38	46.8	88	11	1
Mar 03	18.4	7.1	3.10	28.6	64	25	11
Apr 03	0.2	9.3	5.33	14.8	1	60	39
May 03	29.8	22.2	1.57	53.5	55	42	3
Sep 03[2]	57.1	10.1	1.30	68.5	83	15	2
Oct 03[2]	57.9	8.4	1.25	67.5	86	12	2
Nov 03	55.2	10.5	ND	65.7	84	16	ND
P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
SED[3]	2.03	1.40	0.390	2.69	22.83	23.70	15.97

[1] Only one bulk sample of HT, no Huia samples.

[2] Only 1 bulk sample of HT and one bulk sample of Huia.

[3] Standard error of the difference when comparing data within columns.

ND = Not detected.

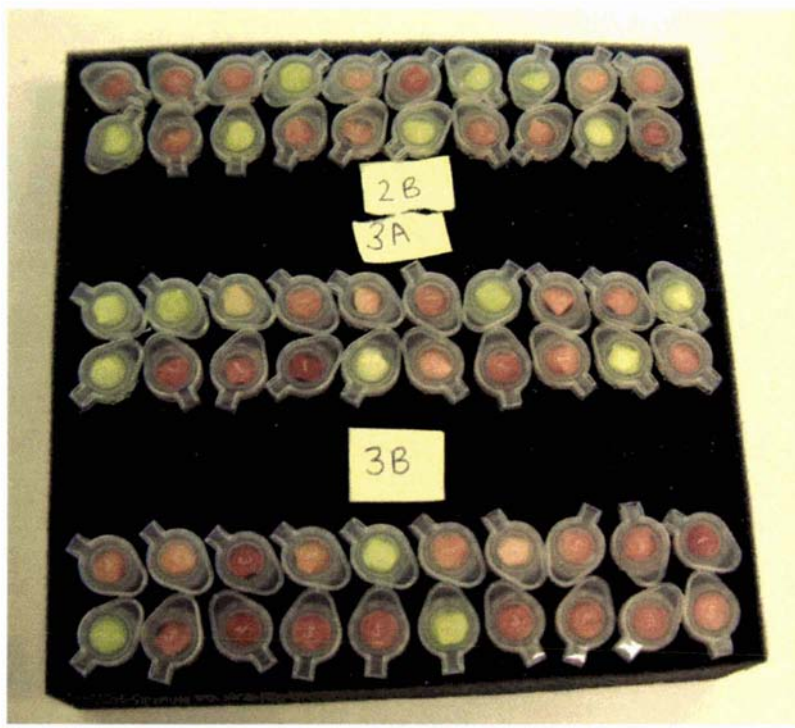
TABLE 3.10 Description of flower heads collected for condensed tannin (CT) analysis and their total CT concentration.

Month	Comments	g CT/kg DM
Mar 02	More brown flower heads than in Nov 01 and Jan 02	39 vs 68 and 75
May 02	Paddocks 1A to 3B more brown flower heads than the rest	41-46 vs 51-66
Oct 02	Very few white flower heads, many green buds	46
Nov 02	Flower heads white, in full bloom	77
Dec 02	Flower heads white, a few starting to brown off	73
Jan 03	50% of flower heads brown	31
Apr 03	Most flower heads completely brown	15
May 03	Flower heads mainly white	54
Oct 03	Mixture of green buds and completely white flower heads	66

3.4.9 Cyanogenesis

Of the 120 leaves tested from each treatment (20 per paddock), 79% of Huia leaves and 78% of HT leaves tested positive for cyanogenesis (Plate 3.3). This indicates that at least 22% of the white clover plants in HT pastures were not HT, since HT is 100% cyanogenic (D. R. Woodfield unpublished data). But contamination could be up to 100%, as other white clover cultivars with a high percentage of cyanogenic plants may have been present.

PLATE 3.3 Results of cyanogenesis testing from one HT (2B) and two Huia (3A and 3B) paddocks showing the change in colour of the filter paper in microcentrifuge tubes from yellow to red for leaves testing positive for cyanogenesis.



3.5 DISCUSSION

This experiment has shown that white clover CT concentrations can be increased by traditional plant breeding. This was achieved primarily through increased flower production with no difference in flower head CT concentration between treatments. However, the increase in flower production resulted in a less productive and persistent plant. Consequently, contamination and competition with weeds and volunteer clover occurred, resulting in a loss of treatment differences in clover CT concentration and stolon mass, and smaller differences in growing point and flower densities, especially from summer 2003 onwards.

3.5.1 Sowing and establishment

The lower density of Huia seedlings during establishment was mainly due to differences in the density of germinable seeds sown (HT was 1.6 times that of Huia). This difference was due to bare seed being sown in HT, as opposed to Superstrike® coated seed in Huia, and was reflected in the density of clover seedlings measured 5 and 11 weeks after sowing (HT was 1.7 times that of Huia).

Harris (1987) showed that the density of white clover plants is equalised by high mortality of plants at high sowing rates, and greater vegetative spread at lower sowing rates. This appears to be the case in this experiment, where stolon growing point densities were similar between treatments at the first measurement, 7 months after sowing.

3.5.2 Stolon growing point density, dry matter yield and weed content

For condensed tannins in white clover to be of use in agriculture, the clover needs to produce enough feed for animals, and to persist long enough to be cost effective. HT clover had good winter and autumn growth, but grew less herbage than Huia in spring and summer, which is a disadvantage for seasonal dairying. Good summer growth of white clover is important for maintaining milk production when pasture quality is declining due to ryegrass flowering and accumulation of dead matter (Woodfield et al. 2001). Leaf size and number per stolon were similar between treatments, so the higher seasonal dry matter yields of Huia over HT were probably due to its higher growing point density.

HT clover has not undergone any selection for agronomic performance and hence may lack adaptation to intensive grazing. Siral white clover, which was used as a parent of HT because of its unusually prolonged flowering, was developed in Australia from an Algerian ecotype. Woodfield & Caradus (1994) compared Siral to Huia under sheep grazing and found lower growing point densities, a lower proportion of clover in the pasture and lower clover DM production for Siral than for Huia. In New Zealand, Mediterranean clovers such as Siral are often winter active and become summer dormant during periods of high temperatures and drought (Caradus 1994). These effects were evident in this experiment when comparing HT to Huia.

Stolons are the primary sinks for carbohydrate storage in white clover (Hart 1987) and therefore support clover persistence in temperate environments. Stolon yields were greater for Huia than HT for the first two measurements, 15 and 17 months after sowing. This difference, and the consistently lower growing point density in HT suggests HT plants are likely to be less persistent than those of Huia. This reduced persistence is evident in the loss of treatment effects in 2003 and the high levels of contamination in HT in January 2004.

3.5.3 Clover morphology

Plant branching order and loss of tap root

The transition of plants from tap rooted to clonal is associated with a decline in productivity and persistence, as plants are smaller and rely on small nodal roots for water and nutrient supply. The faster loss of tap roots in Huia than HT may relate to the former having larger and more complex plants at the first measurement (Brock & Tilbrook 2000).

This loss of tap roots was faster than previously reported by Brock & Tilbrook (2000) and Brock et al. (2000) in sheep grazed perennial ryegrass/white clover pastures. This may be due to greater damage from treading in cow versus sheep grazed pastures, more rapid plant development in monoculture or greater attack by pathogens (Thomas 2003).

The average distribution among branching orders of plants in this experiment (27% first, 47% second, 21% third, 4% fourth, 1% fifth and sixth) was similar to that reported

for Waikato dairy pastures (21% first, 47% second, 25% third, 6% fourth, 1% fifth and sixth) by Harris (1994), but with slightly more first order and less third order plants. Once all plants had assumed a clonal growth form (September 2002), more than 20% of the population was as first order plants, except for Huia in Nov 02. In contrast, Harris (1994) reported more than 20% of the population as first order only from August to December and in February.

First order plants are normally most abundant in spring (Brock et al. 1988; Harris 1994; Pinxterhuis 2000), but were highest in January 2003 in this experiment. As these pastures were sown as monoculture, grazing frequency differed to that in mixed ryegrass/white clover pastures. The lack of grazing in winter and early spring in this experiment may have reduced spring fragmentation, thereby reducing the proportion of first order plants at this time compared with other experiments. White clover stolons also have the potential to initiate branches during mild winters, when competition from other species is minimal (Patterson et al. 1995).

Differences between treatments in flowering and in some cases, stolon length, were evident from January 2002 to January 2003, but disappeared after a dry spell in February 2003. Mortality of HT plants may have been low in the first summer due to the majority of plants still being tap rooted. The higher proportion of first order plants in HT than Huia in spring 2002 may have increased plant mortality over the following summer.

Stolon length, branching and flowering

Differences between treatments in growing point densities were not directly related to the number of branches per plant, per node or per stolon. HT had more intact plants per turf than Huia in November 2001, so despite having less stolons per plant, it had similar growing point densities to Huia. The trend for higher densities in Huia for the remainder of the experiment could not be clearly explained by plant morphology.

The higher flower densities in HT than Huia were due to a greater number of flowers per stolon and per node. Increased flowering can reduce stolon branching (Thomas 1980), as each node has the potential to produce either a flower or a branch. This relationship was not evident in this experiment. The proportion of nodes supporting

either a flower or a stolon branch was always less than 20% for both clovers. However, the flux of birth and death of branches and flowers was not measured, and the percentage of nodes that did branch or flower could therefore have been considerably higher. Newton et al. (1992) noted that 40% of axillary buds may be non-viable. Loss of viability may have had a greater effect on branching than the loss of sites due to flowering, as less than 6% of nodes contained flowers at any one time in both Huia and HT.

Turves used to study white clover morphology were collected in areas that did not have excessive weeds or bare ground, whereas growing point density measurements included all areas. As HT pastures tended to have more weeds in autumn and winter, and more bare ground in summer, growing point densities were lower. Huia had similar numbers of stolons per plant to HT on most occasions, but tended to have more intact plants per turf. It appears that differences between treatments in growing point density were due mainly to better survival of Huia plants. Flowering has long been associated with reduced clover persistence (Gibson 1957), and the shorter internode length of Huia produced denser stolon branching, providing less room for weeds to grow. Differences between the clovers in root characteristics or resistance to invertebrate pests or viruses may have also affected persistence (Woodfield & Caradus 1996) but these were not studied.

3.5.4 Flowering

Despite the higher flower densities in HT than Huia, HT flower densities were low in September and early October, when bloat may be a problem. Siral white clover was used in the breeding of HT to increase flower production, but Grasslands NuSiral, a recently released cultivar selected from Siral for improved seed production, may have been a better parent cultivar. NuSiral is early flowering, and showed a 48% increase in flower density compared to Siral (Ayres et al. 2002). New Zealand experiments involving rotational grazing by sheep or cattle showed average growing point densities of NuSiral were similar to that of Huia (Woodfield et al. 2003).

Similar flowering densities for Huia and HT at the end of the second summer were due to similar numbers of flowers per node. The ratio of flowering to non-flowering nodes is under both genetic and environmental control (Thomas 1987). White clover is as an

outbreeding species, hence even within cultivars considerable morphological and physiological variation exists (Caradus et al. 1993). Reduced flowering in HT pastures may have resulted from better survival of less vigorously flowering genotypes within HT, or from contamination from other clover cultivars post-establishment.

3.5.5 Condensed tannins

Clover CT concentrations were higher in HT than Huia because of the earlier and longer flowering period, and higher flower densities of HT. Flower head CT concentrations were generally similar between treatments. Clover (leaf + flower) CT concentrations were consistently lower than the 20 to 40 g/kg DM reported to be optimal for ruminant nutrition (Waghorn et al. 1990). However, the optimum concentration for animal performance will vary across plant species, as some CT are more astringent than others (Mangan 1988).

Flower head CT ranged from 6 to 94 g/kg DM across all samples analysed. If concentrations could be maintained at the higher end of this range there may be advantages for ruminant nutrition. To be able to maintain high CT concentrations, the reasons for the large variation over time must be determined. A range of biotic and abiotic factors have been reported to affect CT concentrations, with varying responses amongst plant species, plant genotypes and plant parts. CT concentrations may be affected by soil moisture and fertility, temperature, light intensity and carbon dioxide concentration (Briggs 1990; Anuraga et al. 1993; Carter et al. 1999; Gebrehiwot et al. 2002; Michimasa & Kihachiro 2003) but relationships have not been established for white clover. Soil moisture and fertility were not routinely assessed in this experiment, but no relationship was evident between flower head CT concentrations and the application of fertiliser, or with climate.

CT concentrations in this experiment were measured solely in the flower heads, as peduncles contain no CT (Woodfield et al. 1998). Flower heads in this experiment made up 0.34 to 0.60 of the flower dry matter. Whole flower (flower head + peduncle) CT concentrations (flower head CT concentration x proportion of flower as flower head) ranged from 9 to 42 g/kg DM. Stockdale & Dellow (1995) found similar differences in flower CT concentrations over time in irrigated white clover pastures in Victoria, Australia (19 to 46 g/kg flower DM). The high variability in irrigated pastures

indicates soil moisture is not the main factor affecting flower CT concentrations in white clover. Differences could also not be attributed to season, as average CT concentrations in spring/early summer (35 g/kg flower DM) were similar to those in autumn (32 g/kg flower DM).

Free and total CT concentrations appeared to be affected by the stage of flower development, being highest when flowers were in full bloom, and lowest when green buds or senescent flowers were present. Similar variation with stage of plant development has been reported in sainfoin leaves (*Onobrychis vicifolia* Scop.) (Lees et al. 1995), where CT production begins very early in leaf development, and continues until the leaf is fully unfolded and expanded. As the sainfoin leaf begins to yellow, CT declines, with virtually none present at senescence. Lees et al. (1995) speculated that the loss of CT during senescence is due to recycling within the plant, as the synthesis of CT requires substantial metabolic input.

Insect pest populations of clover seed crops in New Zealand peak from late November to early December (White 1990), coinciding with full bloom and the highest flower head CT concentrations in this experiment. Condensed tannins have been implicated as a means of plant defence (Swain 1979). When in full bloom, white clover flower heads are elevated above the leaf canopy, exposing them to herbivore grazing and insect feeding. Less mature flowers have shorter peduncles and are therefore less exposed, reducing the requirement for protection by CT.

It has previously been reported that CT concentrations are high in immature fruit, with a sharp decline as the fruit reaches maturity (Mosel & Hermann 1974). Fruit provide a seed dispersal mechanism for plants (Villee et al. 1989). High CT concentrations in immature fruit may deter animal feeding before seeds are ripe, and the subsequent drop in CT concentrations at maturity would enable seeds to be ingested and therefore dispersed by animals. A decline in CT in senescing white clover flowers (once seeds have developed), may encourage grazing animals to ingest seed heads, providing for natural scarification of the seeds as they pass through the animal's digestive tract, facilitating the spread of viable seeds.

Little research on the proportion or concentration of bound CT in plants has been undertaken. Iason et al. (1995) studied seasonal variation in CT concentration in Yorkshire fog (*Holcus lanatus* L.). As in this experiment, they found an increasing proportion of bound CT with increasing leaf age, with highest concentrations in dead matter. The increasing proportion of fibre bound CT in senescent tissues is probably due to progressive complexation of free CT, and a decrease in the production of CT (Iason et al. 1995).

The lack of difference between treatments in flower head CT, despite differences in the initial testing of the seed line (Figure 2.5), may be due to differences in environmental conditions and management, contamination from buried seed in the field, or differences in sampling and analysis procedures. Seed lines were only tested twice a year prior to use in this experiment, using fully developed flowers with no senescent material. For the field experiment described in this chapter, testing was done more regularly and sampling encompassed all stages of flower development that may have been grazed by cows. The variability introduced by this sampling method may have masked any differences between treatments for flowers at the same stage of development. These differences reiterate the importance of field testing new cultivars under the conditions in which they must perform.

For initial testing of the seed line, birdsfoot trefoil was used as a standard for CT analysis, whereas lotus major was used in this study. CT with a higher procyanidin to prodelphinidin ratio are more reactive using the Butanol-HCl assay (Kraus et al. 2003). White clover CT is 100% prodelphinidin (Jones et al. 1976). Lotus major CT (predominantly prodelphinidin) is therefore more appropriate as a standard than birdsfoot trefoil CT, which is predominantly procyanidin (Foo et al. 1996; 1997).

3.5.6 Contamination of clover pastures with volunteer plants

White clover seeds germinated following the establishment of clover pastures sown in April 2001, with more in HT than Huia. Germination was greatest following a dry period in February 2003, when only 68% of the average monthly rainfall occurred (Appendix 3.5). The proportion of these seedlings that survived to mature plants was not determined, but substantial establishment of white clover from seed after pasture depletion by drought has previously been reported in New Zealand (Harris 1987).

The lack of, or reduced treatment differences in the last 10 months of the experiment, the presence of morphologically different white clover plants, and cyanogenesis in less than 100% of plants in HT pastures indicated the presence of white clover of different genotypes to that sown. This experiment was sown into paddocks that had previously been used for dairying. Dairying soils in New Zealand contain large amounts of buried white clover seed (3-91 kg/ha; Suckling & Charlton 1978; Ledgard et al. 1988), which can remain viable for at least 30 years (Suckling & Charlton 1978). Cultivation can bring this seed to the soil surface (Hill et al. 1999), where it may germinate. New pastures will contain a mixture of plants arising from sown seed, buried seed, natural reseeding, seed transfer in animal dung, and plants surviving spraying and cultivation (Burggraaf & Thom 2000). It is therefore likely that the benefits of new cultivars displaying particular traits may be diluted by the presence of plants from different sources and with different traits to those sown.

Ledgard et al. (1990) showed the white clover component from buried seed in a dairy pasture within 5 km of this experimental site increased from about 10% to 37% over one year from sowing, as estimated from characteristics of clover stolons. Their study site had only 11 kg/ha of buried white clover seed. Dodd et al. (2001) studied the contribution of 3 oversown white clover cultivars to clover populations in summer dry hill pastures where the resident vegetation had been removed. Eighteen months after sowing, the sown clover cultivars contributed 43 to 82% of the total white clover population, reducing to between 27 and 56% after another 3 years. The conditions in their experiment are likely to have been worse for white clover survival than in this experiment, however, the gradual recovery and eventual domination of resident white clover genotypes over introduced genotypes suggests any benefits of HT clover may not be maintained in the long-term.

Incorporation of novel traits into white clover would require the use of well-adapted cultivars as a carrier. Huia white clover was used as base material in the breeding of HT. Although this cultivar has widespread adaptation, most recent cultivars are better adapted to rotational grazing by cattle (van den Bosch et al. 1986; Woodfield et al. 2001, 2003). Any future breeding efforts should use germplasm well adapted to the conditions in which it needs to perform, and it should be thoroughly tested under these conditions before commercial release.

3.6 CONCLUSIONS

HT produced more flowers than Huia and therefore had higher clover plant CT concentrations, despite flower head CT concentrations being similar. Clover plant CT concentrations were low compared to other legumes containing concentrations beneficial to ruminants, but animal testing is required to determine any potential benefits, as different plant species have CT with different biological effects. A better understanding of the large variation in floral CT between sampling dates may enable the development of clover pasture management to maintain maximum CT concentrations.

The increased flower production in HT led to a reduced stolon growing point density and therefore lower herbage production, poorer plant persistence, and a higher proportion of weeds in the pasture. These pastures were also invaded by volunteer clover genotypes, resulting in few treatment differences in the last 10 months of the experiment. To gain any benefits from future clover breeding for increased CT concentrations, more persistent germplasm must be used.

CHAPTER 4

The performance of dairy cows grazing high floral tannin white clover

CHAPTER 4: The performance of dairy cows grazing high floral tannin white clover

4.1 ABSTRACT

The effects of high tannin (HT) white clover on dairy cow performance was evaluated in five short-term grazing experiments over two lactations. Ten mixed age Friesian cows (five of which were rumen fistulated to allow measurement of rumen digestion) grazed monocultures of either Huia or HT white clover at an unrestricted allowance. Pasture condensed tannin (CT) concentrations were low, but significantly different between treatments in four out of the five experiments, ranging from 0.6 to 5.0 g/kg DM in Huia pastures and 0.6 to 12.0 g/kg DM in HT pastures. Mild bloat occurred in cows grazing both treatments, even when CT concentrations were higher than those predicted to prevent bloat. Rumen ammonia concentrations were high in cows grazing both treatments, but were 5 to 26% lower in cows grazing HT than for those grazing Huia, indicating reduced proteolysis. Rumen pH was also consistently higher in HT cows, but effects on rumen soluble protein concentrations were inconsistent and total rumen volatile fatty acid concentrations were not affected. The small differences in pasture CT concentrations resulted in small effects on rumen digestion and no treatment differences in milk production or composition.

This chapter forms the basis of a paper published in: ***Proceedings of the New Zealand Grassland Association 66*****:** 221-226. (Appendix 4.1).

4.2 INTRODUCTION

White clover (*Trifolium repens* L.) is a desirable component of pastures for animal production in New Zealand. This is attributed to its high protein and low structural fibre concentrations, with positive consequences for intake, digestion and utilisation in ruminants (Ulyatt 1980; Thomson 1984). In fresh pasture diets, approximately 70% of protein is degraded to ammonia by rumen microbes (Waghorn & Barry 1987), with soluble protein generally more rapidly and completely degraded than insoluble protein (Chalupa 1984). Undegraded plant protein and microbial protein flowing out of the rumen form the major protein source for the animal (National Research Council 2001).

With good quality forage diets a high proportion of rumen ammonia is incorporated into microbial protein, but about 30% of plant nitrogen is lost as ammonia absorbed through the rumen wall (MacRae & Ulyatt 1974), and after conversion to urea in the liver is largely excreted. The proportion of plant nitrogen lost as urea depends on the crude protein concentration of the diet, its degradability in the rumen, and the availability readily fermentable energy. Lower crude protein concentrations result in lower nitrogen losses (Beever et al. 1985). Cohen (2001) fed dairy cows irrigated clover dominant pastures and estimated the energy required to excrete excess nitrogen could have produced 0.5-2.0 kg more milk per cow per day.

Feeding cattle on pastures containing white clover can lead to bloat. Bloat develops when gas produced during fermentation of feed is trapped within stable foam in the rumen (Jones & Lyttleton 1969). This foam prevents normal expulsion of rumen gases, and as a consequence, ruminal volume and intraruminal pressure increase, which in severe cases can rapidly lead to death. Soluble plant proteins contribute to the formation of rumen foam (McWilliam 1974). The ability of condensed tannins to precipitate plant proteins (Kendall 1966) accounts for the absence of bloat in cattle fed forages containing sufficient concentrations of these compounds.

CT also benefit the protein nutrition of ruminants. CT are released from plant cells when chewed, and bind to and protect plant protein from microbial degradation in the rumen, increasing the flow of plant proteins to the intestines for absorption (Waghorn et al. 1987b). The effects of CT depend on its structure, concentration and plant source (Barry et al. 2001), with CT in some plants inhibiting rumen fermentation (Barry &

Manley 1984), reducing intakes (Barry & Duncan 1984) and limiting amino acid absorption from the intestines (Waghorn 1996). CT in the legume birdsfoot trefoil (*Lotus corniculatus* L.) are beneficial to dairy cows, increasing milk production by 14% to 20% in mid to late lactation (Woodward et al. 1999; 2000). Similar results were found in sheep, with no effects evident in early lactation (Wang et al. 1996a). In some cases, the extra protein reaching the intestines may also increase milk protein concentrations, and milk fat concentration may be reduced (Woodward et al. 2000).

White clover contains CT in its flowers. HT white clover (see Section 2.4.4) has a higher herbage CT concentration than Huia white clover because of higher flower densities (Chapter 3). This study evaluates whether this can improve protein utilisation and prevent bloat in dairy cows. Cows grazing HT or Grasslands Huia white clover at different stages of lactation are compared for voluntary feed intake, rumen digestion, milk yield, milk composition, and bloat incidence and severity.

4.3 MATERIALS AND METHODS

4.3.1 Experimental design

Pure clover pastures of Huia and HT white clover were established at the Dexcel Lye Farm, Hamilton, New Zealand in autumn 2001. Details of the site, pasture establishment and management are described in Chapter 3. The research was conducted as a series of short term grazing experiments, covering two lactations. Experiments ran from 19 November to 15 December 2001 (Dec 01), 21 January to 16 February 2002 (Feb 02), 11 March to 6 April 2002 (Apr 02), 10 November to 6 December 2002 (Dec 02) and 24 March to 10 April 2003 (Apr 03).

Ten lactating multiparous Friesian dairy cows (5 with rumen fistulae) were assigned to each treatment (Huia or HT clover) group. In Apr 03, only 4 fistulated cows were available for each treatment. Where possible, the same cows were used in each experiment, but were allocated to treatments separately for each experiment. Treatment groups were balanced for age and current milk production. Cows within treatment groups were used as replicates, and milk and rumen data collected during each uniformity period (see Section 4.3.2) were used for covariate analysis. Following a

bloat observation period at the start of each experiment, cows were drenched to prevent bloat (Section 4.3.9), because bloat may reduce feed intake and interfere with animal performance (McClymont 1973).

4.3.2 Grazing management

Each 27-day experiment consisted of a 9-day uniformity period, followed by an 18-day treatment period. During the uniformity period all cows grazed together on perennial ryegrass/white clover pastures on an unrestricted allowance (approximately 50 kg DM/cow/day). During the treatment period, cows were split into two herds; one herd grazing each white clover treatment (HT or Huia), on an unrestricted allowance (ranging from 40 to 65 kg DM/cow/day across experiments, Table 4.1). In Apr 03 the treatment period was reduced to 12 days, due to a limited feed supply. Cows were given a new pasture break every 24 hours, after the morning milking. Paddocks of each clover type were grazed in rotation (Appendix 4.2). Each paddock (6 per treatment) was divided into 3 breaks (except in Apr 03 when only 2 breaks per paddock were possible), so that measurements at the end of each experiment covered all paddocks. Cows were back-fenced onto each break using portable electric fences, and had access to water at all times.

4.3.3 Pre and post-grazing herbage mass

Pre and post-grazing herbage mass was estimated by cutting eight 0.2 m^2 quadrats to ground level in each break during the last 6 days of each treatment period. The cut herbage was dried at 95°C for 36 hours and weighed. The difference between pre and post-grazing mass was used to estimate average herd intakes using the following equation:

$$\text{Intake (kg DM/cow/day)} = \frac{\left[\text{Pre-grazing pasture mass (kg DM/ha)} - \text{Post-grazing pasture mass (kg DM/ha)}\right] \times \text{Daily break size (ha)}}{\text{Number of cows}}$$

4.3.4 Pasture chemical and botanical composition

A pasture sample was collected by hand-plucking to estimated grazing height from each break grazed during the last 6 days of each treatment period. Grazing height was estimated by visual assessment of previously grazed pasture breaks. Samples were collected between 1 pm and 3 pm on the day before grazing. A 150 g sub-sample was dried at 60°C for 36 hours, before grinding through a 1 mm sieve and analysing by NIRS (Near Infrared Reflectance Spectroscopy) for crude protein (CP), lipid, soluble carbohydrate (CHO), acid detergent fibre (ADF) and neutral detergent fibre (NDF) concentrations and estimated organic matter digestibility (OMD) and metabolisable energy (ME) content (Corson et al. 1999). A separate 150 g sample was dried at 95°C for 36 hours, then weighed to determine DM%.

A further sub-sample was used to determine pasture botanical composition. Samples were dissected into each species and dead material. White clover leaves were dissected into leaflet and petiole, and flowers into flower head and peduncle (Figure 2.1). Herbage samples were dried at 95°C for 24 hours, before weighing and calculating the proportions of species and clover components on a DM basis.

4.3.5 Clover flower head chemical composition

CT concentration in flower heads dissected from pasture samples collected from each paddock before grazing (Chapter 3) was measured by the butanol-HCl colorimetric method (Terrill et al. 1992a). The proportion of CT in the diet (assuming no grazing selection for or against flowers) was calculated from the concentration of CT in the flower heads multiplied by the proportion of flower heads in the corresponding botanical composition sample.

Clover flower head samples collected from each paddock in May 2002 and monthly from November 2002 to May 2003 for CT analysis (Chapter 3) were also analysed for the chemical constituents described in Section 4.3.4.

4.3.6 Voluntary feed intake

The pasture dry matter intake of each cow was estimated during the final 6 days of each experiment using the alkane faecal marker technique of Dove & Mayes (1991). On each of the final 11 days of each experiment, all cows were dosed at 7:30 am and 3:00 pm (before milking) with known quantities of synthetic dotriacontane (C_{32}) alkane faecal marker (304 to 356 mg/capsule). In April 2002, gelatine encapsulated alkanes were not available, and C_{32} tablets were used. After an equilibration period of 5 days, faecal samples (approximately 200 g wet) were collected per rectum immediately before each milking for 6 days. The faecal samples from each cow were bulked, and stored frozen (-18°C) until the end of the experiment, when a composite sub-sample from each cow was dried at 60°C for 4 days, then ground through a 1 mm sieve.

Three representative pasture samples (A, B, C) were collected from each of the last 6 daily pasture breaks of each treatment in each experiment (coinciding with collection of faecal material). Samples were collected by taking 20 hand plucks pre-grazing to estimated grazing height, before bulking across the 6 days, providing 3 representative pasture samples per treatment (A, B, C). These were then sub-sampled, freeze-dried and ground through a 1 mm sieve before alkane analysis.

Faecal and pasture alkane concentrations were determined by the procedures of Mayes et al. (1986) using an automated GLC (5890A; Hewlett-Packard, Avondale, PA). Pasture intakes of each cow were calculated using the following equation of Dove & Mayes (1991) for each of the three pasture alkane profiles (A,B,C) for each treatment in each experiment:

Intake (kg DM/cow/day) = Dose C_{32}/(Pasture C_{31} x (Faeces C_{32}/Faeces C_{31})-Pasture C_{32})

Where Dose C_{32} is the dose rate (mg/day) of C_{32}, Pasture C_{31} and Faeces C_{31} are the concentrations (mg/kg of DM) of hentriacontane (C_{31}) alkane in pasture and faeces, respectively, and Pasture C_{32} and Faeces C_{32} are the concentrations (mg/kg of DM) of C_{32} alkane in pasture and faeces. C_{31} was used as the reference marker in preference to tritiacontane (C_{33}), because the latter is present in very low concentrations in white clover.

4.3.7 Rumen fluid characteristics

Sample collection and pH determination

Fistulated cows were rumen sampled on the final 2 days of each uniformity period, and on 3 of the final 6 days of each treatment period. Samples were taken from each cow 3 times per day; immediately before the morning milking (Time 1; 0730 h), 2 hours after the commencement of grazing (Time 2; 1030 h) and before the afternoon milking (Time 3; 1500 h). On each occasion, a handful of rumen digesta was removed from the centre of the rumen and gently squeezed through cheesecloth, and the expressed fluid collected. Rumen pH was determined immediately after removal of the rumen sample by immersion of the electrode of a pH meter (Ecoscan series pH 5, Eutech Instruments Ltd) in the rumen at the sampling site. The pH meter was calibrated before each sampling time.

Sample preparation and analysis

Separate 1 mL samples of rumen fluid obtained for ammonia, soluble protein and volatile fatty acid (VFA) analysis were centrifuged (HERMLE Z160M microlite centrifuge) for 15 minutes at 14000 rpm. Samples for ammonia analysis had 15 μL of concentrated hydrochloric acid added before centrifuging to retain ammonia. The supernatant from each sample was stored frozen (-18°C) until analysis, except for Dec 02 and Apr 03 soluble protein samples, which were refrigerated at 4°C and analysed within 24 hours of collection. This change in sample storage procedure was implemented because after analysis of Dec 01, Feb 02 and Apr 02 samples it became apparent that freezing samples reduced protein solubility (MacRae et al. 1975).

Acidified ammonia samples obtained in Dec 01, Feb 02 and Apr 02 were analysed by Alpha Scientific Laboratory using a Hitachi 717 wet chemistry auto-analyser (Boehringer-Mannheim), according to the method of Chaney & Marbach (1962). Atypical results from Apr 02 samples prompted reanalysis of ammonia samples at Massey University, using a Cobas FARA II autoanalyser (Cobas Faras, Hoffman-La Roche Ltd, Switzerland) by the procedure of Neely & Phillipson (1988).

Soluble protein was analysed on a Roche Cobas FARA II autoanalyser by Massey University for the first 3 experiments, using the colorimetric procedure of Bradford

(1976), and by the same method for the remaining samples by the Dexcel Nutrition Laboratory. All VFA samples were analysed by gas chromatography (Playne 1985).

4.3.8 Milk yield and composition

Individual cow milk yields were measured electronically with in-line milk meters at each milking. During each experiment, individual cow milk samples were collected twice daily (at each milking) over the final 6 days of the treatment period, and on 2 days at the end of the uniformity period. Milk samples were analysed for fat, protein and lactose concentration using an infrared milk analyser (Milkoscan 133B, Foss Electric, Hillerød, Denmark). In addition, milk samples from Dec 01, Feb 02 and Apr 02 were sub-sampled and analysed for milk urea nitrogen concentration (MUN) using a Hitachi 717 autoanalyser (Roche) by the kinetic UV method (Tiffany et al. 1972). MUN is an indicator of hepatic urea production (National Research Council 2001).

4.3.9 Bloat

In dairy cows, bloat usually occurs 60 to 120 minutes from the start of a rapid eating period, often after the morning milking (Johns 1954). In each experiment the incidence and degree of bloat was measured on the first 3 days cows grazed the clover treatments. Both treatment herds were put on clover pasture at the same time after their morning milking, and removed for bloat scoring half an hour after the first sign of bloat, or at the end of the observation period (2.5 hours) if no bloat was observed. Bloat severity was scored by palpation of the left flank on a scale of 0 to 5 (0, no bloat; 1, mild; 2, medium; 3, moderate; 4, severe; 5, dead) (Appendix 4.3; Johns 1954). All cows were then orally drenched with 30 mL paraffin oil (Whiterex 307, Mobil NZ Ltd) and returned to clover pastures. Following the afternoon milking all cows were drenched with 30 mL paraffin oil to prevent bloat in the evening. Paraffin oil was used to control bloat because it has a short activity period (Laby 1975) and does not inhibit bloating the next day.

For the remainder of each treatment period cows were drenched twice daily with Bloatenz Plus (95% polypropylene oxide polyethylene oxide copolymer; Ecolab Ltd, New Zealand) at 4 mL per cow in 20 mL water, for prolonged bloat protection. Cows were also observed for the first 2 hours of grazing after each milking to ensure cow safety. Any cases of bloat were recorded and bloated cows were drenched with Bloatenz Plus at the manufacturers recommended rate and returned to pasture once the

bloat had subsided.

4.3.10 Rumen foam volume and persistence

Branine & Galyean (1990) defined subclinical bloat as the presence of foam in the rumen, without evidence of rumen flank distension. Rumen foam volume and persistence was measured on five rumen fistulated cows in each treatment immediately after bloat scoring (after 2.5 hours of morning grazing), but before drenching. Foam volume was measured on the final 2 bloat scoring days in Feb 02, and on all 3 bloat scoring days in Apr 02 and Dec 02. No measurements were made in Dec 01 or Apr 03. Three to 4 kg of rumen digesta was collected from each rumen fistulated cow. A 100 g sub-sample was dried in a 95°C oven for 3 days to determine rumen digesta DM%. The remaining sample was squeezed through cheesecloth into a bucket. Foam settling on top of the liquid was scooped off and transferred into a 2 L measuring cylinder. The volume of the foam and any liquid settling out of it was measured at 0, 30 and 60 minutes after transferring to the cylinder.

4.3.11 Statistical analysis

True replication would have required large resources of land, cows and labour. Therefore, individual cows were treated as replicates, while recognising the lack of independence within treatment groups, because all cows grazed together. All data were analysed using the statistical package Genstat 5.

For each cow, milk and intake data were averaged over 6 days, and rumen digestion data over 3 days for each experiment. Data from each treatment period were adjusted using covariate data collected during the uniformity period before analysis, except for rumen data because the covariate was not significant. Adjusted means and standard errors of the difference (SED) between treatment means are presented. Rumen data were also pooled across treatment periods and analysed for differences between treatments at each sampling time, and the average across sampling times. Bloat data could not be analysed because of its low incidence.

Triplicate alkane pasture samples for each treatment within experiments were pooled to obtain an estimate of pasture sample means and variance. SED's for intake estimations for each treatment period were calculated using estimates of the variance between cows

and between pasture alkane samples.

Analysis of variance was used to compare rumen foam, pasture botanical composition, pasture quality, intake estimation by herbage disappearance, and condensed tannin concentration between treatments. Linear regressions of alkane profiles against the proportion of clover flowers in the pasture were performed in Microsoft Excel and significance was tested by linear regression analysis with Genstat 5.

4.4 RESULTS

4.4.1 Pasture characteristics

Average pre-grazing herbage mass ranged from about 1900 to 3900 kg DM/ha across treatment periods (Table 4.1), being highest in December, and decreasing over summer/autumn. The same trend occurred for post grazing herbage mass. Plates 4.1 and 4.2 show the pastures, in particular the density of flowers, in Dec 02 before grazing.

TABLE 4.1 Average pre and post-grazing herbage mass (kg DM/ha) and daily herbage allowance (kg DM/cow/day) of Huia and HT white clover pastures for each treatment period.

Period	Pre-grazing herbage mass		Post-grazing herbage mass		Herbage allowance
	Huia	HT	Huia	HT	
Dec 01	3779	3889	2750	2788	63
Feb 02	3515	3488	2177	2069	58
Apr 02	2504	2414	1195	1038	40
Dec 02	3931	3873	2964	2944	65
Apr 03	1950	1935	1085	1103	48

PLATE 4.1 Cows grazing Huia white clover in December 2002.

PLATE 4.2 Cows grazing HT white clover in December 2002.

Pastures fed in the treatment period of all experiments contained a minimum of 85% clover (Table 4.2), with no significant differences between treatments. The remainder comprised dead matter (up to 6% of the pasture), weeds and grasses. Weeds were negligible in the first two treatment periods, but by Apr 02 both treatments included annual poa (*Poa annua* L.), chickweed (*Stellaria media* L.), redroot (*Amaranthus retroflexus* L.) and broad-leaved dock (*Rumex obtusifolius* L.), despite the application of herbicide (Chapter 3). In Dec 02 the principal weeds were sow thistle (*Sonchus oleraceous* L.), and in some paddocks, broad-leaved dock. In Apr 03 the pastures also included summergrass (*Digitaria sanguinalus* L.), dandelion (*Taraxacum officinale* Weber) and chickweed.

The proportion of flower head in the pasture (calculated from % clover and % clover as flower head in Table 4.2) offered to HT cows was 10% in Dec 01 and Feb 02, but rose to 15% in Apr 02, 16% in Dec 02, then dropped to 5% in Apr 03. The proportion of flower head in Huia pastures ranged from 3 to 10% of DM. The proportion of flower as flower head was similar between treatments, but increased from December to April in both years (Table 4.2), as pre-grazing herbage mass decreased. The proportion of leaf as leaflet, and flower as flower head ranged from 52 to 66% and 40 to 66% across experiments, and did not differ between treatments.

Plucked clover contained 242 to 285 g CP/kg DM across treatment periods, with NDF concentrations less than 290 g/kg DM. The clover was always succulent, with 11 to 16% DM. Predicted ME ranged from 11.3 to 12.0 MJ/ kg DM and OMD was 80 to 85%. The maximum pasture CT concentration was 12 g/kg DM (HT, Dec 02) and the minimum was 0.6 g/kg DM (both clovers, Apr 03).

There were small differences in the chemical composition of the two clovers (Table 4.3). CT concentrations were greater for HT than for Huia in all treatment periods except Apr 03. HT contained slightly lower CP concentrations than Huia, reaching significance ($P < 0.05$) in Dec 01 and Apr 02. There were no significant differences in DM%, NDF, ADF, OMD or soluble carbohydrate concentrations between treatments for any experiment. Predicted ME tended to be lower in HT than Huia, but this was only significant in Dec 02.

TABLE 4.2 Botanical composition (% of DM) of Huia and HT white clover pastures for each treatment period.

Period		% clover	% dead	% non-sown species	% clover as flower	% flower as flower head	% clover as flower head
Dec 01	Huia	98	2.0	0	7.8	40	2.8
	HT	97	2.6	0	21.8	48	10.5
	SED	0.89	0.89		3.06	7.2	0.72
	P	0.599	0.599		0.044	0.371	0.009
Feb 02	Huia	99	1.0	0	11.4	55	6.3
	HT	99	0.8	0	18.1	58	10.5
	SED	0.49	0.49		1.21	2.46	1.09
	P	0.702	0.702		0.031	0.367	0.061
Apr 02	Huia	92	5.7	2.7	17.6	66	11.3
	HT	85	6.3	9.0	29.6	59	17.7
	SED	2.77	3.35	1.65	0.667	5.90	0.54
	P	0.132	0.867	0.064	0.003	0.395	0.007
Dec 02	Huia	100	0.0	0.0	15.6	43	7.1
	HT	98	0.2	2.2	32.8	49	16.0
	SED	1.99	0.20	1.78	3.83	6.43	2.41
	P	0.344	0.423	0.336	0.047	0.440	0.067
Apr 03	Huia	96	0.04	3.8	5.3	55	3.2
	HT	92	0.08	8.4	8.5	61	4.9
	SED	2.62	0.02	2.61	1.49	3.50	1.16
	P	0.223	0.237	0.224	0.17	0.229	0.286

CT concentrations in flower heads ranged from 13 to 79 g/kg DM, but were similar between treatments (Table 4.3). Clover flower heads contained similar concentrations of fibre to the whole plant, but lower CP concentrations (137 to 204 g/kg DM), organic matter digestibility and soluble carbohydrate concentrations (Table 4.4). HT flower heads had lower concentrations of crude protein and lower organic matter digestibility than Huia, but NDF concentrations were similar (Table 4.4).

TABLE 4.3 Chemical composition (g/kg DM unless otherwise stated) of Huia and HT white clover pastures, and CT concentration in flower heads (floral CT; g/kg DM) during each treatment period.

Period		DM %	Pasture CT	ADF	NDF	CP	ME (MJ/kg DM)	Lipid	OMD	CHO	Floral CT
Dec 01	Huia	11.3	1.8	208	278	284	12.03	37	849	120	65
	HT	12.6	7.4	209	277	271	11.95	38	846	125	70
	SED	0.69	0.5	4.0	2.3	2.3	0.04	0.0	2.7	2.6	2.3
	P	0.21	<0.001	0.83	0.58	0.03	0.20	<0.001	0.23	0.23	0.18
Feb 02	Huia	15.7	4.8	193	269	277	11.93	40	844	130	70
	HT	15.6	7.4	196	267	258	11.83	40	839	138	79
	SED	0.46	1.0	3.8	3.3	10.0	0.05	1.0	6.0	4.8	2.6
	P	0.78	0.035	0.44	0.53	0.15	0.18	0.89	0.47	0.22	0.08
Apr 02	Huia	16.6	4.2	192	260	278	11.22	39	807	122	39
	HT	15.1	7.0	204	269	261	11.03	39	795	123	40
	SED	0.77	1.4	6.9	7.6	6.2	0.14	0.5	9.8	5.4	6.8
	P	0.19	0.017	0.13	0.24	0.03	0.22	0.35	0.27	0.98	0.90
Dec 02	Huia	15.2	5.0	182	241	271	11.70	39	830	143	71
	HT	16.1	12.0	198	250	242	11.42	39	806	139	75
	SED	0.73	1.1	6.3	7.3	9.5	0.06	1.0	1.0	2.8	2.8
	P	0.36	<0.001	0.127	0.34	0.09	0.04	1.00	0.81	0.27	0.30
Apr 03	Huia	15.1	0.6	199	275	271	11.40	38	815	132	17
	HT	14.5	0.6	204	284	263	11.28	37	807	131	13
	SED	0.24	0.2	6.1	6.7	7.9	0.07	0.7	6.5	0.7	1.2
	P	0.11	1.00	0.48	0.34	0.42	0.22	0.84	0.37	0.46	0.50

Abbreviations: DM% = dry matter percent, CT = condensed tannin, ADF = acid detergent fibre, NDF = neutral detergent fibre, CP = crude protein, ME = metabolisable energy, OMD = organic matter digestibility, CHO = soluble carbohydrate, SED = standard error of the difference between treatments, P = probability that the treatments are the same.

TABLE 4.4 Chemical composition (g/kg of DM unless otherwise stated) of flower heads of Huia and HT white clover pastures from May 2002 to May 2003.

Month		ADF	NDF	OMD	ME[1]	Lipid	CP	CHO
May 02	Huia	261	231	760	10.7	40	178	102
	HT	260	233	759	10.7	41	172	104
	SED	3.4 ^{NS}	5.0 ^{NS}	5.3 ^{NS}	0.07 ^{NS}	0.8 ^{NS}	2.8†	2.4 ^{NS}
Nov 02	Huia	218	183	796	11.4	42	179	130
	HT	224	178	781	11.2	42	157	136
	SED	4.3 ^{NS}	7.8 ^{NS}	3.7**	0.08*	0.4 ^{NS}	3.0***	3.6 ^{NS}
Dec 02	Huia	238	206	789	11.3	42	186	112
	HT	241	200	775	11.1	41	159	122
	SED	1.2*	3.9 ^{NS}	2.7***	0.03***	0.5 ^{NS}	6.0**	2.9**
Jan 03	Huia	281	270	767	11.0	37	182	97
	HT	289	288	747	10.7	38	165	101
	SED	3.0*	8.9†	4.4**	0.06 **	0.7†	3.7**	3.1 ^{NS}
Feb 03	Huia	251	209	768	11.1	37	153	135
	HT	255	216	749	10.8	37	137	138
	SED	2.2†	5.2 ^{NS}	3.3***	0.04***	0.5 ^{NS}	3.3**	2.5^{NS}
Mar 03	Huia	265	237	744	10.6	37	187	113
	HT	267	246	731	10.4	39	173	117
	SED	3.0 ^{NS}	7.4 ^{NS}	6.4†	0.09†	0.6*	4.8*	2.0†
Apr 03	Huia	294	301	699	9.9	37	204	104
	HT	300	312	683	9.7	37	187	111
	SED	6.6 ^{NS}	9.1 ^{NS}	9.8 ^{NS}	0.1 ^{NS}	0.4 ^{NS}	6.7*	3.5†
May 03	Huia	236	209	761	10.9	41	183	115
	HT	238	209	752	10.8	41	173	119
	SED	1.6 ^{NS}	2.5 ^{NS}	2.9*	0.05*	0.5 ^{NS}	2.0***	2.1 ^{NS}

[1]ME measured in MJ ME/kg DM. For abbreviation definitions see Table 4.3.

^{NS} = not significant, † = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ for differences between treatments.

4.4.2 Alkane profiles of pastures and voluntary feed intake

Concentrations of C_{31} and C_{33} alkanes tended to be higher in HT than Huia pastures, except in April, when both treatments were similar (Table 4.5). This difference was most evident in December of both years, coinciding with the greatest differences between treatments in flowering. The C_{33} concentration in clover ranged from about 3 to 20 mg/kg DM and high values appeared to be associated with a high proportion of flowers in the DM, but this relationship was not significant ($P = 0.372$; Figure 4.1). However, there was a significant correlation between percentage of pasture DM as flower and concentration of C_{31} alkane ($P = 0.007$; Figure 4.2).

Although pluck samples were taken by the same operator for alkane and other analyses, there was considerable variation between replicate samples in alkane concentrations. This may be a result of differences in the proportion of flowers in each sample.

Calculation of pasture intake using the alkane technique requires an adequate alkane concentration in pasture and accurate determination of concentrations in plucked herbage and in faeces. Dove & Mayes (1991) recommended using natural C_{33} alkane and dosed C_{32} alkane for estimating intake, due to better faecal recovery of longer chain alkanes. Both clovers contained low concentrations of C_{33} compared to C_{31} (Table 4.5), so estimates of intakes were based on the shorter C_{31} plant alkane with dosed C_{32}.

Intakes were calculated using C_{31} and C_{32} in faeces and in forage from each of the three samples to illustrate the impact of variation in forage alkane concentrations (Table 4.5). In Apr 02 a new formulation of C_{32} alkane tablets was used. Subsequent *in vitro* testing showed the tablets failed to dissolve rapidly, indicating a likelihood of inaccurate intake predictions for that treatment period. Intakes predicted from pasture replicates within treatment periods were highly variable (Table 4.5) and cast doubt on the validity of all predictions.

Average intakes calculated by the alkane technique (analysed with variation among pasture samples taken into account) were compared with intakes estimated by herbage disappearance (Table 4.6). Intakes based on herbage disappearance suggested high values for both Huia and HT diets, with no effect of treatment.

TABLE 4.5 C_{31} and C_{33} concentrations (mg/kg DM) of Huia and HT white clover pasture samples, and average feed intake (kg DM/cow/day) calculated using each C_{31} alkane pasture sample (A, B, C), for each treatment period.

Period	Treatment	Pasture C_{31}			Pasture C_{33}			Feed intake		
		A	B	C	A	B	C	A	B	C
Dec 01	Huia	30	26	29	3.6	3.2	3.4	21.2	22.5	21.5
	HT	105	53	72	8.7	4.9	6.6	11.9	24.4	17.8
Feb 02	Huia	81	104	82	15.4	17.8	14.2	19.5	14.6	18.8
	HT	113	169	109	16.4	20.3	15.0	18.3	11.8	19.7
Apr 02[a]	Huia	72	104	95	7.8	12.4	9.4	21.9	15.1	16.5
	HT	91	110	90	9.6	10.4	8.5	24.0	19.1	23.5
Dec 02	Huia	56	87	79	6.0	7.7	7.9	23.1	15.1	16.7
	HT	156	139	117	12.6	11.1	11.1	11.7	13.2	15.8
Apr 03	Huia	41	37	53	6.2	5.0	6.2	20.6	23.6	15.6
	HT	38	48	54	5.8	7.1	8.5	27.8	21.3	18.7

[a]Cows were dosed with alkane tablets rather than capsules.

TABLE 4.6 Average daily feed intake (kg DM/cow/day) for cows grazing Huia or HT white clover calculated using the alkane technique and by herbage disappearance for each treatment period. Standard errors of the differences (SED) between treatments are presented for each intake estimation technique. N=10 cows per treatment for the alkane technique, and the average of 6 days for the herbage disappearance technique.

Period	Average daily intake per cow					
	Alkane technique (C_{31})			Herbage disappearance		
	Huia	HT	SED	Huia	HT	SED
Dec 01	21.7	16.6	3.13	16.7	19.5	1.25
Feb 02	17.3	16.2	3.18	21.5	23.7	3.32
Apr 02[a]	17.4	21.6	3.55	21.2	22.3	1.46
Dec 02	17.7	13.2	3.24	20.6	19.7	3.80
Apr 03	19.9	22.6	3.20	20.8	19.9	2.31

[a] Cows were dosed with alkane tablets rather than capsules.

FIGURE 4.1 Relationship between the percentage of pasture DM as clover flowers and C_{33} pasture alkane concentration. Each data point represents the average of Huia (■) or HT (▲) pastures for one treatment period.

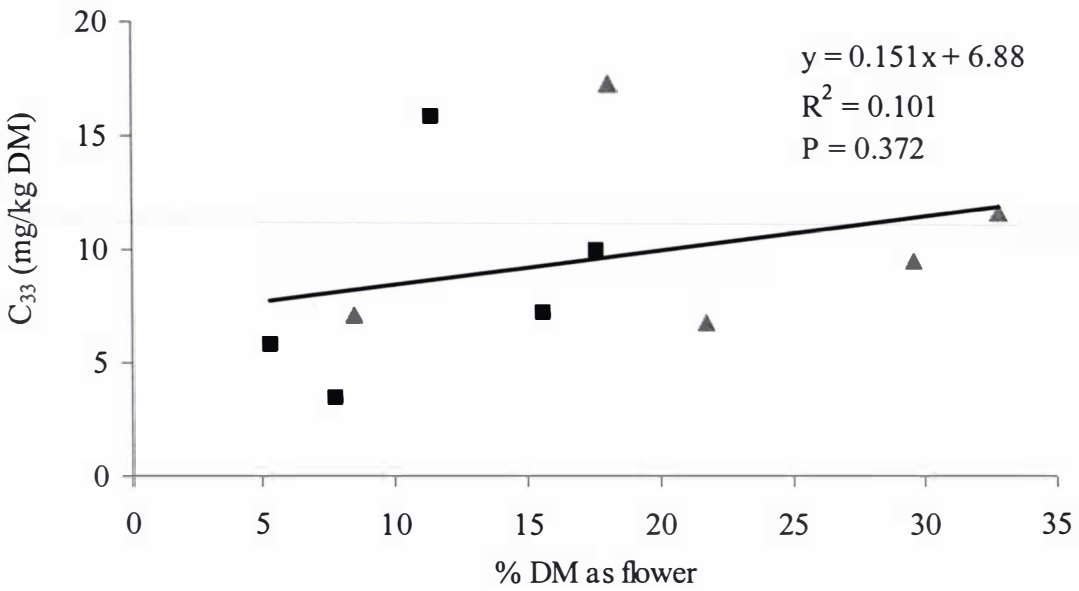
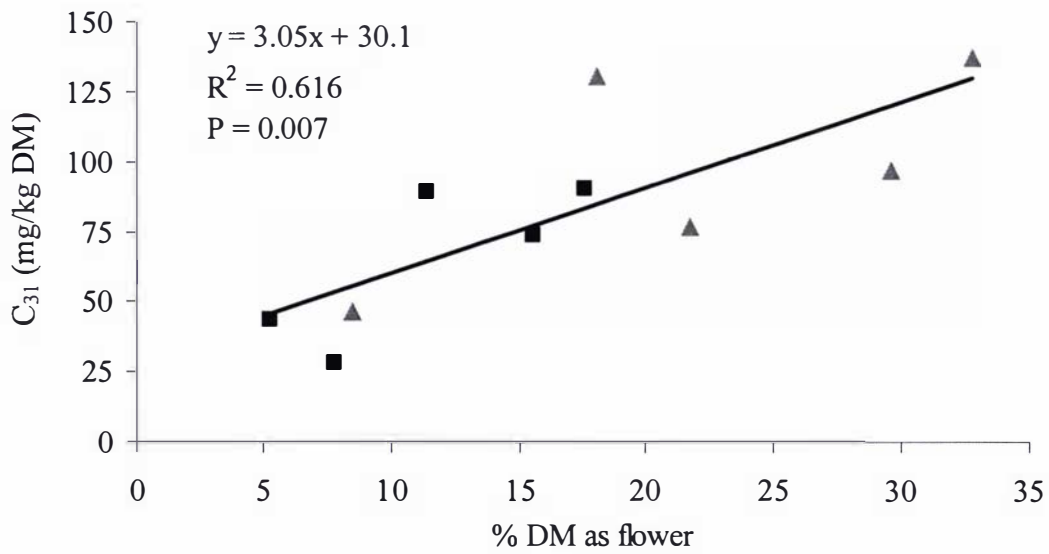


FIGURE 4.2 Relationship between the percentage of pasture DM as clover flowers and C_{31} pasture alkane concentration. Each data point represents the average of Huia (■) or HT (▲) pastures for one treatment period.



4.4.3 Rumen fluid characteristics

In Dec 01 and Dec 02 cows grazing Huia tended towards higher average rumen soluble protein concentrations than those grazing HT ($P < 0.1$; Table 4.7). Concentrations were higher at 2 hours after the start of morning grazing than at either pre-feeding or 6.5 hours after the start of grazing for both treatments in all experimental periods. Soluble protein concentrations tended to be higher in Dec 02 and Apr 03 than for all other treatment periods, probably because of the change in sample storage methods.

Significant ($P < 0.05$) treatment differences in rumen ammonia concentrations (Table 4.8) were detected in all treatment periods except Apr 03. Huia cows had higher rumen ammonia concentrations than HT cows at each sampling time in Dec 01, and for at least one of the sampling times in Feb 02, Apr 02 and Dec 02. Ammonia concentrations increased from a mean of 18.5 mM/L pre-feeding up to 34.9 mM/L 6.5 hours after the start of grazing. Peak concentrations were similar for all treatment periods but higher for cows grazing Huia than HT (37.2 and 32.7 mM/L respectively; $P < 0.001$). Pre-feeding ammonia concentrations (Time 1) were higher in Dec 01 and Dec 02 than in other treatment periods.

VFA concentrations increased from mean pre-feeding values of 132 mM/L to 168 mM/L 6.5 hours after the start of grazing (Table 4.9). VFA concentrations were high in Dec 01 (peak concentrations > 200 mM/L). The only treatment difference was at Time 3 in Dec 02, when total VFA concentrations in Huia fed cows were lower than for HT and lower than for all other Time 3 samplings in all treatment periods (Table 4.9).

There were no consistent treatment effects on the concentration (Appendix 4.4) or molar percentages (Table 4.9) of any VFA. The VFA pool comprised 57-64% acetate, 17-22% propionate, 11-15% butyrate and 5-7% minor VFA. The molar percentage of VFA as acetate decreased from 63 to 59% during the day, while that of propionate and butyrate increased. The molar percentage of minor VFA was always greatest 6.5 hours after the start of feeding (Table 4.9). The molar percentage of VFA as acetate tended to be greater in HT than Huia. Differences between treatments in the proportion of VFA were detected in Apr 03, despite an absence of differences in CT concentration between treatments. During this period the ratio of acetate plus propionate to butyrate was lower in Huia than HT ($P < 0.01$).

TABLE 4.7 Average soluble protein concentration (mg/L) in the rumen fluid of cows grazing Huia or HT white clover for each sampling time, and the average of 3 sampling times of each treatment period.

Period	Time[1]	Huia	HT	SED	P
Dec 01	1	179	147	40.7	0.461
	2	227	139	51.9	0.128
	3	154	94	32.4	0.102
	average	186	127	27.7	0.063
Feb 02	1	111	129	26.5	0.516
	2	224	208	51.6	0.762
	3	141	171	34.2	0.404
	average	159	181	15.7	0.192
Apr 02	1	162	235	45.0	0.145
	2	354	402	69.9	0.516
	3	169	131	35.9	0.316
	average	229	256	39.9	0.514
Dec 02	1	302	264	18.9	0.080
	2	373	296	34.5	0.056
	3	298	255	29.3	0.178
	average	329	274	23.8	0.052
Apr 03	1	288	293	8.6	0.582
	2	397	418	27.6	0.487
	3	363	347	15.1	0.313
	average	350	352	10.1	0.783

[1] 1 = 0730 h; 2 = 1030 h; 3 = 1500 h. See section 4.3.7.

TABLE 4.8 Average rumen fluid ammonia concentration (mM/L) of cows grazing Huia or HT white clover at each sampling time within each treatment period.

Period	Time[1]	Huia	HT	SED	P
Dec 01	1	29.5	22.1	2.79	0.029
	2	30.6	21.9	1.64	<0.001
	3	45.1	37.7	1.68	0.002
Feb 02	1	16.3	9.4	2.32	0.018
	2	23.5	17.1	2.46	0.031
	3	34.5	31.5	1.94	0.164
Apr 02	1	11.7	12.3	1.30	0.647
	2	22.8	24.9	1.27	0.143
	3	35.7	28.7	2.29	0.016
Dec 02	1	27.6	21.6	1.43	0.003
	2	26.3	22.8	1.01	0.009
	3	31.2	30.2	1.26	0.453
Apr 03	1	17.9	16.9	1.52	0.519
	2	23.3	24.0	0.87	0.408
	3	39.3	35.3	1.75	0.063

[1] 1 = 0730 h; 2 = 1030 h; 3 = 1500 h. See section 4.3.7.

TABLE 4.9 Total volatile fatty acid concentration (mM/L), and molar percentage of individual and minor VFA[1], and acetate to propionate and (acetate+butyrate) to propionate ratios in the rumen fluid of cows grazing Huia or HT white clover.

Period	Time[2]	Total VFA		% Acetate		% Propionate		% Butyrate		% Minor		Acetate:Propionate		(Acetate+Butyrate):Propionate	
		Huia	HT	Huia	HT	Huia	HT	Huia	HT	Huia	HT	Huia	HT	Huia	HT
Dec 01	1	173	173	63.0	63.9	18.0	17.8	12.7	12.4	6.35	5.90	3.51	3.61	4.22	4.30
	2	172	153	62.8	62.8	18.8	19.0	12.7	12.2	5.91	5.89	3.36	3.33	4.04	3.98
	3	211	205	60.4	60.4	19.2	19.8	13.3	13.5	6.95	6.43*	3.15	3.06	3.85	3.73
Feb 02	1	133	127	63.0	63.8	18.0	17.2	12.0	12.6	7.00	6.71	3.54	3.74	4.22	4.46
	2	136	132	61.2	61.8	19.4	18.6	12.6	12.4	7.01	6.91	3.14	3.30	3.82	3.99
	3	154	159	58.0	58.6	20.0	20.2	13.8	14.0	7.63	7.22	2.88	2.88	3.58	3.56
Apr 02	1	122	116	65.4	66.2*	17.4	16.8	11.4	11.4	5.63	5.58	3.74	3.94†	4.40	4.62
	2	138	138	60.4	60.9	20.7	22.1†	12.7	12.7	5.68	4.79	2.94	2.72	3.56	3.32
	3	179	159	58.2	59.2	20.4	20.6	15.4	14.4†	5.77	6.08	2.82	2.88	3.53	3.57
Dec 02	1	108	116	61.0	61.8	19.0	19.0	14.4	13.4*	5.56	5.45	3.22	3.22	3.98	3.94
	2	121	115	59.8	60.6	19.8	19.8	14.6	13.8	5.44	5.64	3.02	3.08	3.77	3.76
	3	127	156**	58.8	58.2	20.0	20.4	15.2	15.2	6.02	6.04	2.94	2.86	3.70	3.57
Apr 03	1	134	123	62.5	63.3	19.5	19.5	11.8	11.5	5.77	5.83	3.20	3.28	3.82	3.87
	2	131	131	60.0	61.0	21.8	20.8*	13.0	12.0†	5.85	5.99	2.78	2.98*	3.37	3.54**
	3	138	159	56.5	58.8**	21.8	21.0*	14.8	13.5†	7.00	6.85	2.60	2.78*	3.49	3.61**

[1] Isobutyrate, valerate, isovalerate; [2] 1 = 0730 h; 2 = 1030 h; 3 = 1500 h. See section 4.3.7.
† = $P < 0.1$; * = $P < 0.05$; ** = $P < 0.01$ for differences between treatments within rows.

TABLE 4.10 Average rumen pH of cows grazing Huia or HT white clover for each sampling time within each treatment period.

Period	Time[1]	Huia	HT	SED	P
Dec 01	1	5.75	5.79	0.12	0.756
	2	5.76	5.85	0.07	0.210
	3	5.48	5.74	0.09	0.016
	average	5.66	5.79	0.09	0.154
Feb 02	1	5.93	6.35	0.16	0.026
	2	5.93	6.25	0.15	0.060
	3	5.61	5.86	0.12	0.072
	average	5.82	6.17	0.11	0.017
Apr 02	1	6.32	6.62	0.12	0.035
	2	6.10	6.28	0.13	0.208
	3	5.74	5.94	0.09	0.051
	average	6.05	6.28	0.10	0.063
Dec 02	1	5.81	5.93	0.10	0.243
	2	5.83	5.99	0.07	0.054
	3	5.79	5.87	0.07	0.314
	average	5.81	5.93	0.08	0.146
Apr 03	1	5.96	6.12	0.04	0.011
	2	5.80	6.02	0.06	0.003
	3	5.66	5.78	0.04	0.035
	average	5.81	5.99	0.04	0.007

[1] 1 = 0730 h; 2 = 1030 h; 3 = 1500 h. See section 4.3.7.

Mean rumen pH was 5.66 to 6.28 across all periods, and was lower in Huia than HT cows for at least one sampling time in each experimental period (Table 4.10). Average pH across experimental periods for Huia and HT were 5.95 and 6.16 pre-grazing ($P < 0.001$), 5.88 and 6.09 2 hours after the start of grazing ($P < 0.001$), and 5.66 and 5.84 6.5 hours after the start of grazing ($P < 0.001$), respectively. Pre-grazing pH tended to decrease with increasing herbage allowance. Rumen pH decreased between each sampling time within days, except in Dec 02 (Table 4.10). Cows in both treatments showed signs of heat stress during this period (drooling and panting) and stood under shade for much of the day not grazing, but some cows were grazing in the morning before being taken off the paddock for milking.

4.4.4 Milk production and composition

Daily milk yields averaged 20.7 to 25.0 kg/day in December, and 15.4 to 19.4 kg/day in February and April (Table 4.11). However, there were no significant ($P < 0.05$) differences between treatments in milk, milksolids, fat or protein yields (Appendix 4.5) in any treatment period. In April 2002 milk lactose yield was 9% higher in HT than Huia (0.81 versus 0.74 kg/cow/day, respectively, $P = 0.09$), and corresponding milk yields were 8% higher ($P = 0.12$), but there were no consistent trends across treatment periods.

Milk protein concentrations ranged from 3.3 to 3.8%, fat concentrations from 4.0 to 4.5% and lactose concentrations from 4.6 to 4.9. Treatment did not affect ($P < 0.05$) the percentage of any of the milk components averaged over the 6 days of milk sampling (Table 4.11). However, in Feb 02, protein % was consistently lower in HT cows, reaching significance on 1 out of 6 days of milk testing (3.36 versus 3.47 for HT and Huia, respectively; SED = 0.044; $P < 0.05$). This trend also occurred in Dec 02. Milk urea N concentrations were measured in Dec 01, Feb 02 and Apr 02, and treatment period averages were between 23 and 32 mg/dL, with no effect of treatment (Table 4.11).

TABLE 4.11 Covariate adjusted average daily milk and milksolids (fat plus protein) yields (kg/cow/day) and concentrations of protein, fat, lactose and urea nitrogen (MUN) in the milk of cows grazing Huia or HT white clover for each treatment period.

	Huia	HT	SED	P
Milk yield				
Dec 01	21.4	20.7	0.772	0.391
Feb 02	17.8	18.4	0.774	0.505
Apr 02	15.4	16.6	0.793	0.124
Dec 02	25.0	24.3	0.664	0.289
Apr 03	19.4	18.6	0.719	0.262
Milksolids yield				
Dec 01	1.49	1.46	0.053	0.498
Feb 02	1.32	1.33	0.040	0.773
Apr 02	1.25	1.32	0.050	0.232
Dec 02	1.93	1.86	0.067	0.342
Apr 03	1.55	1.52	0.052	0.533
Protein %				
Dec 01	3.35	3.37	0.048	0.647
Feb 02	3.43	3.34	0.047	0.081
Apr 02	3.65	3.61	0.047	0.340
Dec 02	3.57	3.45	0.071	0.125
Apr 03	3.67	3.77	0.092	0.293
Fat %				
Dec 01	4.04	4.04	0.146	0.991
Feb 02	4.00	4.02	0.122	0.868
Apr 02	4.51	4.49	0.104	0.812
Dec 02	4.22	4.20	0.104	0.912
Apr 03	4.33	4.44	0.149	0.460
Lactose %				
Dec 01	4.82	4.85	0.027	0.419
Feb 02	4.85	4.87	0.047	0.684
Apr 02	4.82	4.81	0.042	0.725
Dec 02	4.64	4.66	0.028	0.474
Apr 03	4.82	4.80	0.045	0.768
MUN (mg/dL)[1]				
Dec 01	31.9	31.2	1.887	0.713
Feb 02	25.6	25.2	0.731	0.579
Apr 02	23.7	23.6	0.922	0.584

[1]MUN was not measured in Dec 02 or Apr 03.

4.4.5 Bloat

The incidence of bloat was low in both treatments. No bloat occurred during the three days of bloat evaluation at the start of each treatment period, but some mild cases occurred in February and April towards the end of treatment periods, despite twice daily drenching. Bloat occurred in both treatments, even with pasture CT concentrations of up to 9.2 g/kg of DM (Table 4.12). All incidences of bloat occurred between 1.5 and 2 hours of the start of the morning grazing. Pasture CP concentrations were between 260 and 290 g/kg of DM on the days that bloat occurred, but were no different to days when bloat did not occur. In Feb 02, on day 18 of the treatment period, 3 cows bloated, as opposed to all other bloat days when only 1 case of bloat was recorded out of the 20 cows. On this day, cows arrived back in the paddock at 10 am (1.5 to 2 hours later than normal), due to a breakdown of the milking machine.

TABLE 4.12 Occurrence and degree of bloat during treatment periods, and concentration (g/kg of DM) of condensed tannin (CT) in pasture breaks where bloat occurred.

Period	Treatment day	Cow number	Treatment	Bloat score	CT
Feb 02	13	8648	Huia	1.0	5.1
Feb 02	15	8148	HT	0.5	4.4
Feb 02	18	8148	HT	1.0	9.2
Feb 02	18	8642	Huia	0.5	2.9
Feb 02	18	8648	Huia	0.5	2.9
Apr 02	16	5707	HT	1.5	4.9
Apr 03	12	5817	Huia	0.5	0.5

4.4.6 Rumen foam volume and persistence

While rumen sampling in Dec 01, it was noted that the rumen contents of cows fed Huia appeared more 'foamy' than those fed HT. One Huia cow expelled approximately 10 L of foaming rumen contents when its rumen cannula was opened at Time 2, on days 15 and 17, despite no external signs of bloat. This happened on a day when delays occurred in bringing the cows to the paddock after milking. Rumen foam volume and persistence was determined in subsequent measurement periods to detect signs of sub-clinical bloat. In Feb 02, a different cow fed Huia also expelled foaming rumen contents.

Bloating potential as measured by rumen foam volume and stability did not show treatment differences. Foam volumes varied between cows (Appendix 4.6), and failed to show much breakdown after 1 hour (Table 4.13). The DM% of rumen contents of all cows was similar in each treatment period, but the volume of foam produced was lower in Dec 02 (corresponding to the absence of bloat in Dec 02) than in Feb 02 and Apr 02.

TABLE 4.13 Volume and persistence of rumen foam collected from cows 2.5 hours after the start of grazing HT or Huia white clover at 0, 30 and 60 minutes after separation from rumen digesta.

Period	Treatment	Digesta	mL foam per kg rumen digesta		
		DM%	0 minutes	30 minutes	60 minutes
Feb 02	Huia	13.5	54	52	51
	HT	13.3	41	40	39
Apr 02	Huia	13.8	60	42	40
	HT	13.5	63	55	50
Dec 02	Huia	12.3	19	15	12
	HT	12.1	19	14	12

4.5 DISCUSSION

The feeding trials with HT clover were designed to measure cow performance, including rumen indicators, as well as pasture characteristics under grazing. Although cow performance was not affected by the higher concentrations of dietary CT in HT pastures, there were some effects on rumen function, suggesting reduced proteolysis. Ruminal changes appeared to be too small to affect a measurable response in milk production or composition.

4.5.1 Pre-grazing herbage mass and diet quality

Herbage allowances, designed to provide cows with an unrestricted feed supply, were based on guidelines for maximising intakes from rotationally grazed ryegrass/white clover pastures (Holmes et al. 2002a). A daily allowance of 40 to 60 kg DM/cow, with at least 2500 kg DM/ha pre-grazing and a post-grazing residual of at least 1600 kg DM/ha was achieved in all treatment periods except Apr 02 and Apr 03. Pre and/or post-grazing herbage DM was lower than guidelines for maximising pasture intake on these occasions.

Adequate herbage mass was difficult to achieve in late lactation (April), as leaf size and petiole elongation decrease with decreasing day length, light intensity and temperature (Mitchell 1956; Brougham 1962; Arnott & Ryle 1982). The proportion of flower as flower head in the DM also increased with decreasing pre-grazing herbage mass (Tables 4.1 and 4.2) because of shorter peduncles rather than larger flower heads. Hence, short day length increased the proportion of flower heads. In contrast, long days (December) resulted in larger leaves and a low proportion of flower heads in the pasture DM, despite high flower densities (Chapter 3). Dilution of flower heads, and therefore CT, by leaves and peduncles with high pre-grazing herbage mass has important implications for maximising dietary CT by grazing management. Assuming beneficial effects of CT for animal performance, management would require grazing intervals to maximise flower density with the minimum herbage mass required to adequately feed cows.

The feeding value of the white clover pastures was high across all treatment periods. CP concentrations exceeded recommendations of 180 g/kg DM for cows producing 20 kg milk/day, or 240 g/kg DM for cows producing 30 kg milk/cow/day; Kolver 2000).

However, NDF was always below the 350 g/kg DM recommended for cows grazing high quality pasture (Kolver 2000).

The CT concentration in the DM ranged from 0.6 to 5.0 g/kg for Huia, and 0.6 to 12 g/kg for HT. Assuming the intake of CT resembled that sampled, these values were well below values of 20-40 g/kg DM considered beneficial for ruminant nutrition (Barry et al. 1986; Waghorn et al. 1987b; Barry 1989; Waghorn et al. 1990; Aerts et al. 1999), but higher than 5 g/kg DM suggested by Li et al. (1996) for bloat prevention.

The proportion of flower heads in the pasture appeared to be the major factor influencing pasture quality. Proportions of dead matter, weeds, and ratios of both leaflet to petiole and flower head to peduncle were similar between treatments. The higher pasture CT concentration in HT than Huia was due to a higher proportion of flower heads. CT concentrations in flower heads were similar for both clovers (Chapter 3). The lower CP concentrations in HT than Huia is a consequence of much lower CP concentrations in flower heads (14-20 g/kg DM) compared with whole pasture (24-28 g/kg DM).

4.5.2 Rumen fluid characteristics

Rumen pH results from a balance between VFA concentrations and salivary buffering (Pitt et al. 1996). Cows fed HT clover had higher rumen pH values than cows fed Huia in each treatment period, but HT was not associated with lower VFA concentrations than Huia, so an increased salivation may have resulted from the CT in HT clover. Landau et al. (2000) reported increased salivation in heifers dosed with quebracho (*Aspidosperma quebracho* Schlecht.) CT and suggested the astringency of CT reduced eating rate with an increased salivation production. Similar suggestions were made by Waghorn (1996), and a reduced intake rate could also minimise diurnal variation in VFA production. Impacts of low pH on fibre digestion (de Veth & Kolver 1999, 2001) would have been minor with these diets because NDF was less than 30% of DM.

Rumen ammonia concentrations were high in both treatments (mean of all measurements of 25.7 mM/L), compared to values from the same cows grazing ryegrass/white clover pastures (average of 16.0 mM/L across all uniformity measurements). The ammonia concentration in rumen contents of cows fed HT clover

were 5-26% lower (treatment period means) than for Huia, suggesting reduced proteolysis. This is small compared to reductions of 30-70% associated with forages containing 30-80 g CT/kg DM (Waghorn et al. 1999). Protein degradation also contributes to the minor rumen VFA (isobutyrate, valerate, isovalerate) which are derived from deamination of amino acids (van Soest 1982), of which concentrations tended to be lower in cows fed HT than Huia in Dec 01 and Feb 02. These data indicate a small effect of CT on rumen proteolysis, but the higher rates of ammonia absorption associated with higher rumen pH (Hoover & Miller 1991) may have also contributed to lower ammonia concentrations in HT cows.

CT can precipitate proteins (Jones & Lyttleton 1971), and this has been associated with lower ruminal protein degradation rates when CT are present in forages (McNabb et al. 1996). Waghorn & Jones (1989) demonstrated 1.7 g CT/kg DM (vanillin estimation technique) was able to lower soluble protein concentration *in vivo*, but effects in this experiment were inconsistent. The lower concentrations of soluble protein in digesta taken from cows in Dec 01, Feb 02 and Apr 02 compared to Dec 02 and Apr 03 were attributable to freezing before soluble protein determination. The procedure did not affect the comparison of the two clovers.

There were no meaningful treatment effects on the percentage of the major VFA or A:P or (A+B):P ratios or VFA concentrations. High VFA and ammonia concentrations and low ruminal pH pre and post-feeding suggest a consistent and active fermentation.

4.5.3 Bloat

Cows fed fresh pastures containing 15% or more of DM as legumes are prone to bloat (Waghorn 1988). The development of bloat is the result of a combination of plant, climate and animal factors. Although it is difficult to predict when it will occur, it is well established that large diurnal fluctuations in feed availability (and rumen pH) can contribute to bloat (Majak et al. 1995). A high gas production is a prerequisite for bloat (Clark & Reid 1974), which may originate from active fermentation and/or a high pre-feeding pH enabling CO_2 release upon acidification from VFA production (Waghorn 1991). The cows were fully fed in these experiments, and the small difference in pre-feeding ruminal pH for the two treatments is unlikely to have affected CO_2 production.

Bloat occurs when ruminal gases become trapped in stable and persistent foam. Good correlations have been reported between the formation of foams *in vitro* from leaf extracts and the incidence of bloat on pastures of the same plant species (Pressey et al. 1963; Kendall 1964; Cooper et al. 1966; Jones et al. 1970). Rumen foam volume and persistence in this research were not affected by dietary treatments, but the experiment with the highest foam volumes also had the highest incidence of bloat.

Bloat foams are based on soluble plant proteins (Mangan 1959), and dietary CT precipitate and lower the concentrations of plant proteins in the rumen. HT clover was expected to affect the rumen environment. A minor impact on rumen ammonia concentrations was evident, but CT concentrations or astringency were insufficient to affect the soluble protein concentration, quantities of rumen foam or the incidence of bloat.

Stockdale (1994) reported a marked reduction in the incidence of bloat when white clover contained more than 5% of DM as flowers, and this was attributed to the effect of CT. However, clover flowers may also reduce bloat through reduced feed digestibility and protein concentrations (Stockdale 1999). In these experiments, flowers made up more than 5% of DM for both treatments, and consequently bloat severity and incidence were low. Bloat did not occur from mid November to mid December, when flower densities were highest (Chapter 3), but several cases occurred in February and April, when cows had been grazing clover for 12-18 days. The cows had been receiving twice daily drenches of Bloatenz Plus, which would have reduced the incidence and severity of bloat.

The peak period for bloat deaths in New Zealand is October/November (Reid 1974). The low clover flower densities and low pasture CT concentrations in HT in October (Chapter 3) are unlikely to be adequate for bloat control.

Li et al. (1996) estimated the threshold CT concentration in forage legumes for bloat safety to be between 1 and 5 g/kg DM, and recommended 5 g/kg DM as a breeding objective for bloat safety in forage legumes. This work has been widely cited (Barry & McNabb 1999; McMahon et al. 2000; Singh & Bhat 2001; Waghorn & McNabb 2003), but the efficacy of the suggested "safe" concentration has not been tested in ruminants.

The occurrence of bloat in this experiment at CT concentrations as high as 9 g /kg DM, while cows were being drenched with bloat preventative, indicates the CT concentrations required for bloat prevention with white clover diets are higher than the 5 g/kg DM predicted by Li et al. (1996). Plant species require testing on a case-by-case basis for bloat prevention, due to differences in the concentration and type of CT, the concentration of soluble protein, and the rate of degradation of feed in the rumen.

4.5.4 Voluntary feed intake

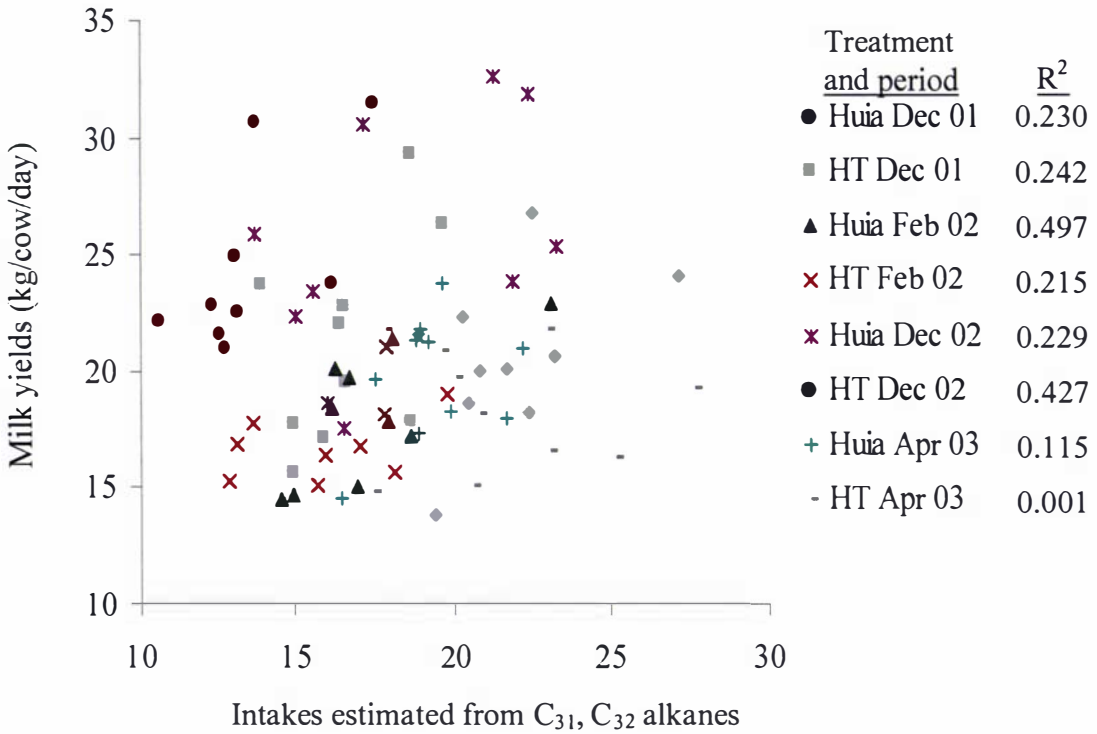
Intakes were estimated using two different techniques, both with shortcomings. The pre and post-grazing herbage mass technique indicates the average intake of all cows in each treatment, rather than individuals. Estimated intakes ranged from 16.7 to 21.5 kg DM/cow/day (mean 20.2) for cows grazing Huia, versus 19.5 to 23.7 kg DM/cow/day (mean 21.0) for those grazing HT (Table 4.6). The accuracy of measuring pre and post-grazing herbage mass will increase as the number of samples collected increases. In practice, the number of samples is largely determined by the number that can be handled with the time and resources available, with many researchers taking between 5 and 12 samples per plot (Frame 1981). The eight samples per pasture break used in these experiments should have been adequate, given that assessments were in monoculture. However, post-grazing herbage mass is more difficult to measure because of its non-uniform nature (Frame 1981).

The use of plant wax alkanes as markers for studies of herbivore nutrition has been widely adopted (Newman et al. 1998), but data obtained from these experiments has not inspired confidence in the technique, despite focussing on the C_{31} plant alkanes, the most abundant in white clover. Mean intakes predicted for all cows over the five treatment periods (Table 4.6) averaged 18.8 (Huia) and 18.0 (HT) kg DM/cow/day, which is 1.4 and 3.0 kg/day lower than herbage cut estimates for the respective forages. Uncertainty was based on two observations; variation in group mean estimates dependent upon which pasture sample (A, B, C) was used for calculations (Table 4.5), and the poor correlations between intakes of individuals based on alkanes versus the milk production of individual cows (Figure 4.3). The disparity in estimates (Table 4.5) from the three forage samples is unacceptable, and prompted a review of alkane concentrations in forage components.

Dove & Mayes (1991) emphasised the importance of herbage samples being representative of material eaten, and Dove et al. (1996) reported white clover flowers contained 16 times the concentration of C_{31} and 11 times the concentration of C_{33} alkanes to that in the leaves. The proportion of clover flower in the pasture was positively correlated with C_{31}, but not C_{33} concentrations in the DM (Figures 4.1 and 4.2).

Dove & Mayes (1991) in a review of the use of alkanes for intake estimation reported average faecal recoveries of 87% for both C_{32} and C_{33}. The largest discrepancy they reported between known intakes of fresh herbage, and intakes estimated using C_{32} and C_{33} alkanes was only 1.7%. In a recent study (K. M. Krause unpublished data) of 6 sheep fed white clover indoors, faecal recovery of naturally occurring odd-chain alkanes was poor. The average recoveries were 73, 95 and 57%, for C_{31}, C_{32} and C_{33}, respectively. Feed intakes were underestimated by approximately 17% when using the alkane technique. These results indicate that the alkane technique is unsuitable for use with white clover diets due to the poor alkane faecal recovery and the difficulty of obtaining a representative pasture sample. The inadequacies of measuring the intake of cows grazing white clover suggests that if intake is one of the most important responses being measured, experiments should be conducted indoors and intakes measured by weighing the feed offered to individual animals and subtracting what is not eaten.

Figure 4.3 Relationship between milk yields (kg/cow/day) of individual cows, and their estimated intake using the alkane (C_{31}, C_{32}) technique (average of three pasture samples per treatment). Data from the Apr 02 treatment period are excluded.



4.5.5 Milk production and composition

Effects of CT concentration and type on milk yield

Few experiments have assessed the effects of CT in forage legumes on milk production. The CT in birdsfoot can increase milk yields from dairy cows by 10 to 20% (Woodward et al. 1999, 2000, 2004). Such results correspond with a similar experiment using sheep, where the CT in birdsfoot trefoil increased milk production by 21% in the second half of lactation (Wang et al. 1996a). In those experiments, CT concentrations ranged from 25 to 45 g/kg DM, which is at least double that in the diet of cows fed HT clover (maximum of 12 g/kg DM). Rumen ammonia concentrations in sheep were reduced by 51% by the action of CT (Wang et al. 1996a), whereas in the current experiments the maximum reduction was only 26%, suggesting effects on proteolysis were too small to affect milk yield. Increasing the CT concentration of HT clover may therefore increase milk production.

The effects of CT are also related to their chemical structure (Tanner et al. 1994). The CT of different plant species differ in their structure, and CT concentrations as high as 44 g/kg DM in sulla (*Hedysarum coronarium* L.) did not affect milk yield in sheep in mid to late lactation (Roy et al. 2002). Lotus major (*Lotus pedunculatus* Cav.) CT sub-units are predominantly the same as that of white clover (Jones et al. 1976), and when fed as a sole diet, its CT does not benefit ruminant production (Waghorn & Shelton 1997; Waghorn et al. 1999). Hence, white clover CT may also be of little or no benefit to production.

Reduced milk production has previously been attributed to white clover CT. Stockdale & Dellow (1995) supplemented cows grazing flowering white clover with maize (*Zea mays* L.) silage in spring/summer and autumn. Dietary crude protein concentrations of unsupplemented cows ranged from 170-270 g/kg of DM compared with 150-220 g/kg DM for unsupplemented cows, and was similar between seasons. Responses to supplementation were better in autumn, when pasture CT concentrations averaged 2 g/kg of clover DM, as opposed to spring/summer when CT was 7 g/kg of clover DM. Different milk responses were attributed to the higher CT concentrations in spring/summer inducing a shortage of available nitrogen in the rumen, resulting in inefficient use of the energy in maize silage. However, in the current experiment,

rumen ammonia concentrations were consistently high, and therefore HT was unlikely to have induced a nitrogen shortage.

Poor clover growth in the early spring of 2002 precluded milk production measurements in early lactation. Wang et al. (1996a) showed the CT in birdsfoot trefoil to have no effect on milk yield or composition in early lactation in sheep, but milk yield, and milk protein and lactose yield were increased in mid to late lactation. In the current experiment, flower densities and therefore pasture CT concentrations were low in early lactation (Chapter 3), so HT was unlikely to have affected milk production at this time.

These experiments did not directly assess the effects of clover CT on milk production. HT clover had more flowers than Huia, causing differences in feed quality other than CT concentrations. Although rumen ammonia was lower in HT than Huia, this may be partly due to lower dietary protein concentrations. The ME of HT clover also tended to be slightly lower than that of Huia (Table 4.3). Milk production by dairy cows in New Zealand is limited primarily by the intake of ME (Kolver 2000), so increasing flowering in white clover to increase the CT concentration in the diet may not be of benefit to milk production.

Effects of HT clover on milk composition

Despite HT cows having lower rumen ammonia concentrations than Huia on most occasions, MUN concentrations (above 18 mg/dL; van der Merwe et al. 2001) indicated excessive rumen ammonia concentrations in both treatments. The excess ammonia has a metabolic cost to the cow of about 12 kJ/g NH_3-N converted to urea (Baldwin 1995) and reduces energy available for milk production (Twigge & van Gils 1984).

MUN is correlated with blood urea nitrogen, which in turn has a linear relationship with urinary N excretion (Jonker et al. 1998). Waghorn & McNabb (2003) implicated CT may reduce nitrate leaching and nitrous oxide emissions as a consequence of increasing the proportion of excretory N in faeces versus urine. The high MUN concentrations of cows grazing HT clover indicate urinary urea excretion would be high, and therefore N losses through nitrous oxide formation and nitrate leaching would not be reduced.

The lower protein concentration in the milk of HT cows in Feb 02 was unexpected and

unlikely to be a consequence of diet composition because CP concentrations were similar for both clovers (Table 4.3). HT clover did not affect milk fat concentrations in this experiment. Woodward et al. (2000) found the CT in birdsfoot trefoil reduced milk fat concentrations from cows in mid to late lactation. A decrease in milk fat concentration may result from an increase in the proportion of propionate relative to acetate and butyrate in the rumen (Sutton 1981). However, the ratios of these VFA did not differ greatly between treatments in this experiment.

Wang et al. (1996a) reported from a study of sheep fed a restricted diet of birdsfoot trefoil, that CT increased milk lactose concentration and secretion from mid to late lactation. Cows were fully fed in these experiments and milk lactose concentration was not affected by clover type. Lactose determines milk volume (Holmes et al. 2002c), and is derived from propionate, which did not differ in concentration between treatments in these experiments.

4.6 CONCLUSIONS

HT white clover did not provide any substantial benefits to dairy cow performance above that of Huia white clover when cows were fully fed white clover as a sole diet. The higher CT concentrations and lower CP concentrations in HT were able to reduce ammonia concentration in the rumen, but this did not result in more milk or large changes in milk composition. The incidence and severity of bloat was low in both clover treatments, but HT clover was not able to consistently prevent bloat, even above the theoretical minimum CT concentration for prevention of 5 g/kg DM. CT concentrations in HT were low compared to published information where positive effects on milk production have been detected. Further increases in the concentration of CT in white clover flowers, or increases in the proportion of flowers may improve animal performance.

CHAPTER 5

In vitro digestion of white clover with different proportions of flower and leaf

CHAPTER 5: *In vitro* digestion of white clover with different proportions of flower and leaf

5.1 ABSTRACT

The protein in white clover can be poorly utilised by ruminants because of extensive degradation to ammonia in the rumen. The condensed tannins (CT) in some legumes reduce rumen proteolysis, increasing protein supply to the animal. White clover produces CT in its flowers, and the effects of increasing proportions of clover dry matter (DM) as flowers (and therefore floral CT) on soluble protein, ammonia and volatile fatty acid (VFA) concentrations were determined during *in vitro* incubations. Minced mixtures of 0, 25, 50, 75 and 100% of DM as white clover flower (F) with the remainder as white clover leaf, were incubated *in vitro* for 24 hours and were sampled at 0, 2, 4, 8, 12 and 24 hours. Treatments contained 0, 13, 26, 39 and 52 g CT/kg DM, respectively. A further treatment with 50% flower and 50% leaf had polyethylene glycol added to remove the effects of CT. Increasing the percentage of white clover as flowers from 0 to 100% reduced the net conversion of plant N to ammonia-N from 29 to 12%. This was partly due to reduced solubility of the protein. Treatments with 75% or more of clover DM as flowers reduced ammonia concentrations to levels likely to limit microbial growth, but total VFA production was not affected although the percentage as acetate increased. The contribution of CT to treatment effects was small compared to effects attributed to the difference in chemical composition between flowers and leaves.

5.2 INTRODUCTION

Excessive protein degradation in the rumen of animals fed white clover (*Trifolium repens* L.) lowers its nutritive value. Soluble proteins, released from plants cells as a result of chewing or microbial action, are susceptible to proteolytic degradation, yielding peptides, amino acids and ammonia (NH_3). Rumen bacteria use ammonia to synthesise protein, which can later be digested by the host animal. However, where protein concentrations in feed are excessive, such as in white clover, much of the ammonia produced is absorbed from the rumen and excreted as urea. This wastage can be reduced by either balancing the supply of soluble protein with available energy to increase microbial protein synthesis or by reducing the degradation of protein in the rumen (Hoover & Stokes 1991).

The flowers of white clover contain condensed tannins (CT), which bind to plant proteins when released from plant cells, protecting the protein from microbial degradation. CT lower protein solubility and ammonia concentrations in rumen fluid (Barry et al. 1986; Chiquette et al. 1989) and increase the quantity of non-ammonia nitrogen (NAN) and amino acids reaching the small intestine (Waghorn et al. 1987a,b). White clover forage consists mainly of leaves, with varying proportions of flowers. HT white clover (Section 2.4.4) has a higher proportion of its harvestable DM as flowers than Huia white clover, and therefore a higher forage CT% (Chapter 3).

Cows grazing HT white clover had lower rumen ammonia concentrations than those grazing Huia (Chapter 4), indicating reduced proteolysis. The limited number of treatments meant a relationship between the proportion of clover flower in the diet and rumen digestion could not be defined. Also, the experiment did not separate the effects of CT from other differences in diet composition between the clovers. In order for plant breeders to optimise the CT content of white clover through flowering, the response to a range of dietary flower contents and the contribution of CT to such responses needs to be determined within one clover variety.

This experiment determined the effects of increasing the percentage of clover flowers over a range of 0 to 100% of DM relative to clover leaves on plant protein degradation and the production of energy yielding substrates (volatile fatty acids; VFA), using *in vitro* incubations. The specific effects of CT were assessed by including polyethylene

glycol (PEG) in some incubations, to bind with and inactivate the CT (Jones & Mangan 1977). White clover leaves and flowers were minced to resemble chewing and incubated with buffered rumen fluid to simulate rumen digestion. The products of digestion were measured at intervals over a 24 hour incubation period.

5.3 MATERIALS AND METHODS

5.3.1 Experimental design

In vitro incubations included five ratios of leaf:flower and one including PEG to remove the effects of CT. Eighteen samples of each mixture were incubated, with three replicates removed for sampling at 0, 2, 4, 8, 12 and 24 hours. White clover bred for increased floral condensed tannin (HT clover; Section 2.4.4) was used for all treatments as follows (DM basis):

1. 0% flower, 100% leaf (0F)
2. 25% flower, 75% leaf (25F)
3. 50% flower, 50% leaf (50F)
4. 75% flower, 25% leaf (75F)
5. 100% flower, 0% leaf (100F)
6. 50% flower, 50% leaf + PEG (50F+PEG)

5.3.2 Collection and mincing of clover

Approximately 800 g of clover leaves and clover flowers were harvested from the HT clover swards described in Chapter 3, on 29 January 2003. The clover was 10 to 15 cm long, and was cut to between 1 and 2 cm above ground level using hand shears. Clover was harvested between 1 and 2 pm, and was immediately placed in a plastic bag on ice, then transferred to a -18°C freezer.

Two days after collection, herbage samples were chopped into 2-3 cm lengths (Plates 5.1 and 5.2), for ease of mincing. Samples were kept frozen during this process, and were immediately transferred back into the freezer once chopping was complete. Sixty gram samples of chopped clover flowers and chopped clover leaves were dried for 24 hours at 95°C to determine DM%.

Chopped clover components were mixed in appropriate treatment ratios immediately before mincing on the day before incubation. About 200 g of herbage for each treatment mixture was minced in a Kreft Compact meat mincer R70 (Kreft, Gmbh; Plate 5.3) with 12 mm diameter holes in the sieve plate. The mincer head, screw, cutter and sieve plate were stored in a freezer (-18°C) before use to avoid thawing of the plant material during mincing, and prevent degradation before incubation with rumen liquor. Barrell et al. (2000) reported mincing to be the most effective method of preparing forages for *in vitro* incubations, as it results in a particle size distribution of DM similar to that of chewed forage (Waghorn et al. 1989). Lag periods at the start of incubation are reduced in minced compared to freeze-dried and ground or chopped forages. The mincer was operated by one person and minced material was stored frozen (-18°C) until incubation the following day.

5.3.3 Feed analysis

A sample of fresh clover leaves was dissected into leaflets and petioles (leaf stems) and a sample of fresh clover flowers was dissected into flower heads and peduncles (flower stems) (Figure 2.1). Samples were dried at 95°C to determine their contribution to leaf and flower DM.

A sample of minced clover flowers was freeze-dried and ground through a 1 mm sieve. Free, protein bound, and fibre bound CT concentrations were then determined using the butanol-HCl colorimetric technique (Terrill et al. 1992a) with a lotus major (*Lotus pedunculatus* Cav.) standard. The CT concentration of each treatment was calculated by multiplying the proportion of clover flowers by their CT concentration.

Minced samples of leaf and flower were oven dried at 60°C, ground through a 1 mm sieve and analysed for crude protein (CP), lipid, soluble carbohydrate (CHO), acid detergent fibre (ADF), neutral detergent fibre (NDF), organic matter digestibility (OMD), and metabolisable energy (ME) content by Near Infrared Reflectance Spectroscopy (NIRS) (Corson et al. 1999). The composition of each treatment was calculated from the proportion of flower and leaf used in the mixtures.

5.3.4 Particle size distribution

For each treatment, a sub-sample (15 g wet weight) of minced forage was used to measure particle size distribution by wet-sieving (Turner & Newall Ltd) as described by Waghorn (1986). Minced herbage samples were washed through a stack of 5 counter-rotating sieves (sides of square holes were 4, 2, 1, 0.5, and 0.25 mm) using about 1500 mL water recirculated through the sieves at a flow rate of 4 L/minute for 5 minutes. Material retained on the sieves was transferred onto tared filter paper and dried at 60°C for 24 hours to determine particle dry weight.

Material that passed through all of the sieves was mixed and a 1 L aliquot centrifuged at 2000 x g for 20 minutes. The resulting pellet was transferred to filter paper and dried as above. This fraction is termed 'residues'. The remaining (soluble) material not retained on sieves or as residues was calculated by difference from the dry weight of the initial sample less the sum of recovered particulate matter.

5.3.5 Incubation

Minced herbage samples were weighed into 50 mL schott bottles with vented caps (0.5 g DM per bottle). The bottles were warmed, flushed with carbon dioxide for approximately 40 seconds to remove oxygen, and 12 mL of McDougall's buffer (McDougall 1948; Appendix 5) and 0.5 mL of cysteine sulphide reducing agent (Appendix 5) were added to each bottle. Bottles were capped and placed in an incubator at 39°C (Plate 5.4). For the 50F+PEG treatment, a separate buffer containing 2 g/L PEG (Appendix 5) was used, enabling the CT to bind preferentially with PEG. Immediately following placement of the last bottle in the incubator, 3.0 mL of strained (through cheesecloth) rumen fluid inoculant was added to each bottle. The rumen fluid had been collected and bulked from three cows grazing pasture (Appendix 5) approximately 5 minutes before inoculation. The lid of the incubator was closed, and incubation took place with an oscillation rate of 90/minute.

The pH of rumen fluid inoculum was measured and sub-sampled for VFA, NH_3 and soluble protein determination (Section 5.3.6).

PLATE 5.1 Frozen chopped white clover leaves ready for mincing.

PLATE 5.2 Frozen chopped white clover flowers ready for mincing.

PLATE 5.3 Frozen white clover leaves being minced in a Kreft Compact meat mincer.

PLATE 5.4 Samples in incubator. Spaces indicate removal of samples at 2 hours of incubation.

5.3.6 Incubation medium characteristics

When the bottles were removed from the incubator, the pH was measured using a hand-held pH meter (Ecoscan series pH 5, Eutech Instruments Ltd), calibrated at each sample removal time. Separate 1 mL samples were obtained from each bottle for NH_3, soluble protein and VFA analysis and centrifuged (HERMLE Z160M microlite centrifuge) for 15 minutes at 14000 revolutions per minute (rpm). Samples for NH_3 analysis had 15 μL of concentrated hydrochloric acid added before centrifuging to keep the NH_3 in solution. Supernatants were transferred to 1.5 mL microcentrifuge tubes; samples used for NH_3 and VFA analysis were stored frozen (-18°C) whereas those for soluble protein analysis were held at 4°C and analysed within 24 hours of collection.

Soluble protein concentrations were analysed using the colorimetric procedure of Bradford (1976) with a bovine serum albumin standard. VFA analysis was by gas chromatography (Playne 1985) and NH_3 determinations used a Cobas FARA II autoanalyser (Cobas Faras, Hoffman-La Roche Ltd, Switzerland) and the procedure of Neely & Phillipson (1988).

5.3.7 Calculations

All *in vitro* concentrations of NH_3, VFA and soluble protein were adjusted to account for the contribution of metabolites from the rumen inocula to indicate net production from the herbage mixtures. For example, net ammonia production was calculated after subtracting the 0 hour (h) ammonia concentration from values at 2, 4, 8, 12 and 24 h of incubation, within treatments. Calculations involving soluble protein were adjusted to remove the contribution of soluble protein from the rumen inocula (Table 5.3).

5.3.8 Statistical analysis

pH and net rumen metabolite concentrations were plotted against incubation time for each treatment. Data were subjected to analysis of variance using the model for completely randomised experimental designs in Genstat 5. SED's accounted for the variation in the time zero samples as well as variation at each time period. Differences were treated as significant at $P < 0.05$.

Comparisons were made between treatments for each incubation period, between incubation times, and interactions between treatments and incubation times. Linear,

quadratic and cubic regression analysis was performed on all data using Genstat 5 to determine the response to increasing proportions of clover flower relative to leaf at each incubation time.

5.4 RESULTS

5.4.1 Feed analysis

Leaflet and petiole comprised 71 and 29% of the leaf fraction, respectively, with flower head and peduncle accounting for 66 and 34% of flower DM. Clover leaves had a lower DM% but higher CP concentration than flowers (Table 5.1). Minced clover flowers had 52.4 g CT/kg DM, of which 50% was free, 44% was bound to protein, and 6% was bound to fibre.

TABLE 5.1 Dry matter percentage of white clover leaf (0F) and flower (100F) at harvest, and chemical composition (g/kg DM) of minced clover herbage mixtures.

Treatment	DM	CP	CHO	NDF	ADF	Lipid	CT
0F	16.8	239	140	240	205	41	0.0
25F	17.3	218	142	245	218	39	13.1
50F	18.2	197	145	249	231	37	26.2
75F	18.9	176	147	254	243	34	39.3
100F	19.6	155	149	258	256	32	52.4

For treatment descriptions see Section 5.3.1

DM = dry matter percent, CP = crude protein, CHO = soluble carbohydrates, NDF = neutral detergent fibre, ADF = acid detergent fibre, CT = condensed tannins.

5.4.2 Particle size distribution

Minced clover flowers contained larger particles and less soluble DM than clover leaves (Table 5.2). The largest differences were in particles larger than 2 mm making up 13-22% of the minced material for treatments containing flowers compared to only 6% for the 100% leaf treatment.

TABLE 5.2 Particle size distribution (% distribution in dry matter) of minced white clover mixtures with different percentages as flower (F) and leaf.

Treatment	Particle size						
	> 4 mm	2-4 mm	1-2 mm	0.5-1.0 mm	0.25-0.50 mm	residues	soluble
0F	1.0	5.1	13.3	15.3	23.0	9.2	33.1
25F	6.2	11.9	15.0	15.0	13.5	6.3	32.0
50F	5.9	6.9	11.2	17.6	18.8	10.3	29.4
75F	7.1	6.9	15.3	14.2	16.2	8.2	32.0
100F	9.4	13.1	15.4	12.5	14.8	7.7	27.2

For treatment descriptions see Section 5.3.1

5.4.3 Inocula characteristics and zero hour incubation samples

Characteristics of the rumen fluid inocula are presented in Table 5.3. These characteristics changed on addition to the incubation bottles (after adjusting for dilution by other bottle contents) (Table 5.3). Adjusted ammonia concentrations in the bottles at zero hours incubation were higher than in the rumen inocula. Adjusted VFA concentrations in bottles were also inconsistent with inocula. Soluble protein concentrations and pH are not adjusted and compared to inocula because of release of protein from plant cells during mincing and addition of buffer to bottles.

TABLE 5.3 Characteristics of the rumen fluid (inocula) used for incubations and comparable concentrations in incubation bottles at 0 hours for each treatment. Concentrations for each treatment are adjusted for dilution of rumen fluid by buffer and reducing agent, and assuming 50% of the wet weight of forage mixtures had been released as water from plant cells[1].

Parameter	Inocula	0F	25F	50F	50F+PEG	75F	100F
pH (non-adjusted)	6.03	7.13	7.10	7.10	7.13	7.10	7.11
Ammonia (mM/L)	12.7	13.6	15.6	16.5	17.4	18.6	19.8
Soluble protein (mg/L) (non adjusted)	387	515	448	385	360	287	258
Total VFA (mM/L)	131	168	140	128	101	111	114
Acetate (mM/L)	88	121	94	84	59	62	66
Propionate (mM/L)	23.4	29.9	28.9	26.7	24.4	31.2	30.6
Butyrate (mM/L)	14.7	13.7	13.6	13.6	14.0	14.2	15.0
Minor VFA[2] (mM/L)	5.1	3.1	3.4	3.3	3.3	3.2	3.8

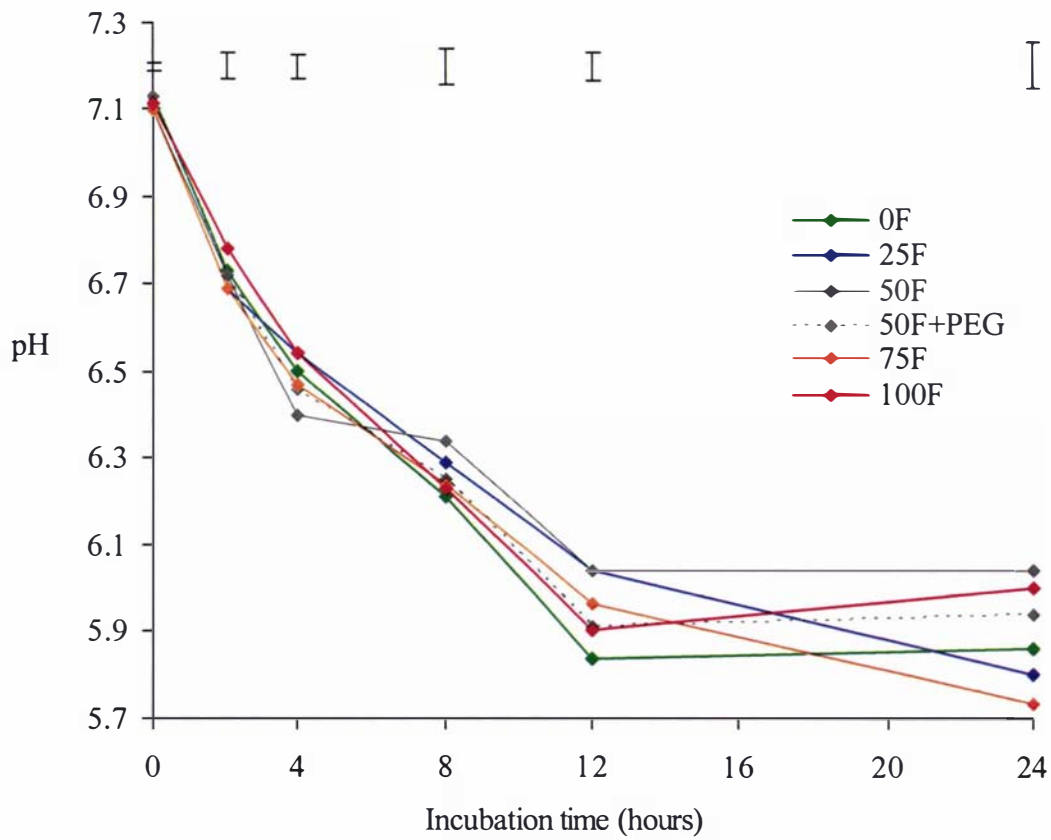
[1] Chewed forage is similar to minced forage, and about 60% of cells are broken by chewing during eating (Waghorn et al. 1989). Given that equilibration between cell contents (from a ruptured cell) and the buffered inocula will take some time, an initial value of 50% is used for the amount of water released from cells into the incubation medium.

[2] Minor VFA = isobutyrate, valerate, isovalerate

5.4.4 Incubation medium pH

There were no significant treatment effects on incubation medium pH over the incubation period (Figure 5.1). The incubation medium of all treatments had a pH of 7.1 at 0 hours, then declined over the first 12 hours of incubation. All treatments maintained a pH greater than 5.7 over the 24 hours of incubation.

FIGURE 5.1 Change in pH of incubation medium during *in vitro* incubation of white clover herbage with different percentages of flower (F) and leaf including one treatment containing PEG. Error bars represent SED's. Treatments are described in Section 5.3.1.



5.4.5 Soluble protein

At 0 h of incubation, soluble protein concentrations were highest for the 100% leaf (0F) treatment (446 mg/L) and declined as the percentage of flower increased, to 189 mg/L for 100F (Figure 5.2). There was a linear relationship ($P < 0.001$) between the percentage of clover leaf and soluble protein concentrations at all times except at 2 hours, when the relationship was quadratic ($P < 0.01$).

During the incubation, soluble protein concentrations in 0F, 25F and 50F declined rapidly over the first 2 hours, and the 0F treatment continued to decline until 4 hours. There were significant treatment differences in soluble protein concentrations at all times except 8 hours of incubation, when values ranged from 210 to 226 mg/L across all treatments. Concentrations of soluble protein in 75F and 100F gradually increased throughout the experiment, but at 24 hours were still significantly lower ($P < 0.001$) than all other treatments (Figure 5.2).

The CT in 50F only reduced soluble protein concentrations by 20% at 2 hours and by 14% at 4 hours of incubation relative to 50F+PEG. 50F+PEG had soluble protein concentrations intermediate to that of 25F and 0F at 2 hours and similar to 25F at 4 hours, after which concentrations were unaffected by CT.

Soluble protein in the incubation medium was less than 7% of forage crude protein (Figure 5.3). At the commencement of incubation (0 h), values were highest for treatments with a high percentage of leaves, but from 8 to 24 hours of incubation values were highest for treatments with a high percentage of flowers (Figure 5.3). The changes in soluble protein pools over the duration of the experiments are presented in Table 5.4. The concentration of soluble protein relative to plant protein was reduced by CT. Values for 50F were 26% less than that of 50F+PEG at 2 hours ($P < 0.001$), and 17% less at 4 hours ($P < 0.01$) of incubation (Figure 5.3).

FIGURE 5.2 Soluble protein concentration of plant origin in the incubation medium from *in vitro* incubations of white clover herbage with different percentages of flower (F) and leaf, including one treatment containing PEG. Error bars represent SED's.

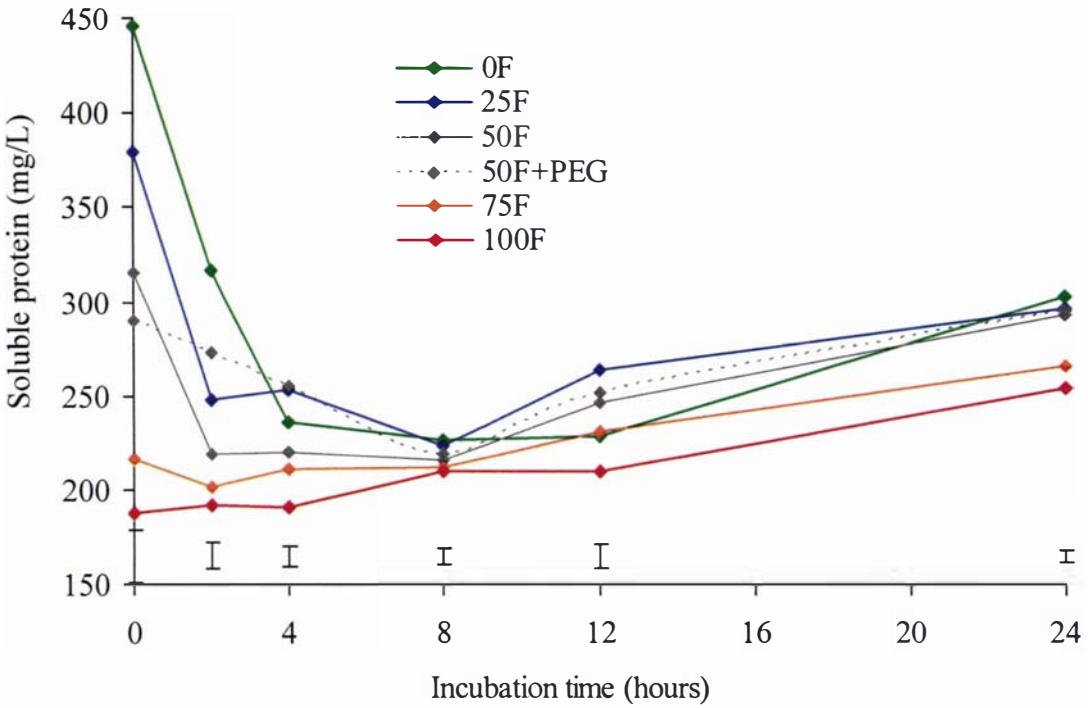


FIGURE 5.3 Quantity of soluble protein expressed as a percentage of plant protein from *in vitro* incubations of white clover herbage with different percentages of flower (F) and leaf, including one treatment containing PEG. Error bars represent SED's.

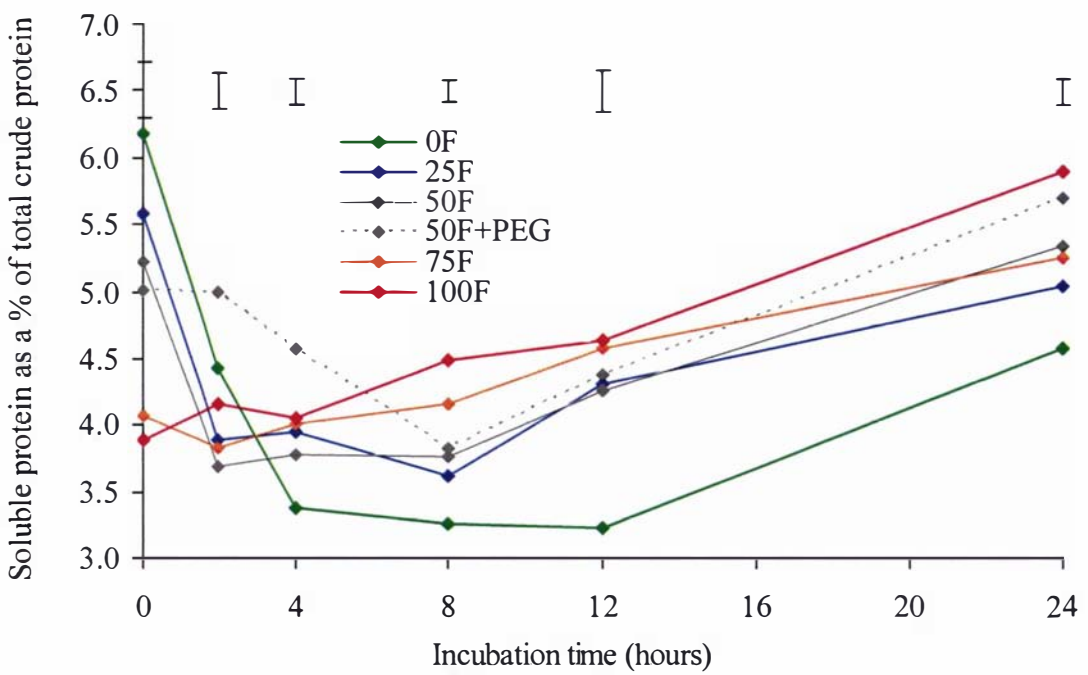


TABLE 5.4 Change in the soluble protein pool (mg soluble protein/g DM/h) during *in vitro* incubation of white clover herbage with different percentages of flower (F) and leaf, with PEG included in one treatment to remove the effects of condensed tannins.

Time	0F	25F	50F	50F+PEG	75F	100F	P linear	P PEG
0-2 h	-2.09	-1.84	-1.51	-0.01	-0.22	0.22	***	*
2-4 h	-1.25	0.06	0.09	-0.42	0.15	-0.08	**	NS
4-8 h	-0.07	-0.18	0.00	-0.37	0.07	0.17	*	*
8-12 h	-0.02	0.38	0.24	0.27	0.19	0.06	NS	NS
12-24 h	0.11	0.06	0.09	0.11	0.06	0.10	NS	NS

For all tables: P linear is the significance of the linear regression for increasing percentages of flower, P PEG is the significance of the difference between 50F and 50F+PEG, NS = non significant, * = $P < 0.05$, ** = $P < 0.01$; *** = $P < 0.001$.

5.4.6 Ammonia

Ammonia (NH_3) concentration in the incubation medium (after correction for input from rumen inocula) represents production from protein degradation less incorporation into microbial protein. All treatments followed the same pattern (Figure 5.4), with highest net production during the first 2 hours (0.06 to 0.10 mM NH_3/g DM/hour; Table 5.5), little change between 2 and 4 hours, a loss from 4 and 8 hours, followed by a steady increase. There was a linear relationship between net NH_3 concentration and percentage of clover leaf for all incubation times ($P < 0.001$). CT in the 50F treatment increased NH_3 concentration by 24% at 2 h ($P < 0.01$) and decreased values by 31% at 12 h ($P < 0.01$) compared to 50F+PEG.

The net conversion of plant N to ammonia N (Figure 5.5, Table 5.6) followed the same trend as ammonia concentrations, with increasing percentages of flower decreasing the rate and extent of plant N appearing as ammonia. Treatments with more than 50% of DM as flowers had very low ammonia concentrations at 8 and 12 hours incubation, suggesting a greater utilisation than production by microbes. Net ammonia released by 24 h of incubation ranged from 12 to 29% of forage N, with highest values for the 0F treatment. CT had little effect on the net conversion of plant N to ammonia N (Figure 5.5, Table 5.6).

FIGURE 5.4 Net ammonia concentration from *in vitro* incubations of white clover herbage with different percentages of flower (F) and leaf, including one treatment containing PEG. Error bars represent SED's.

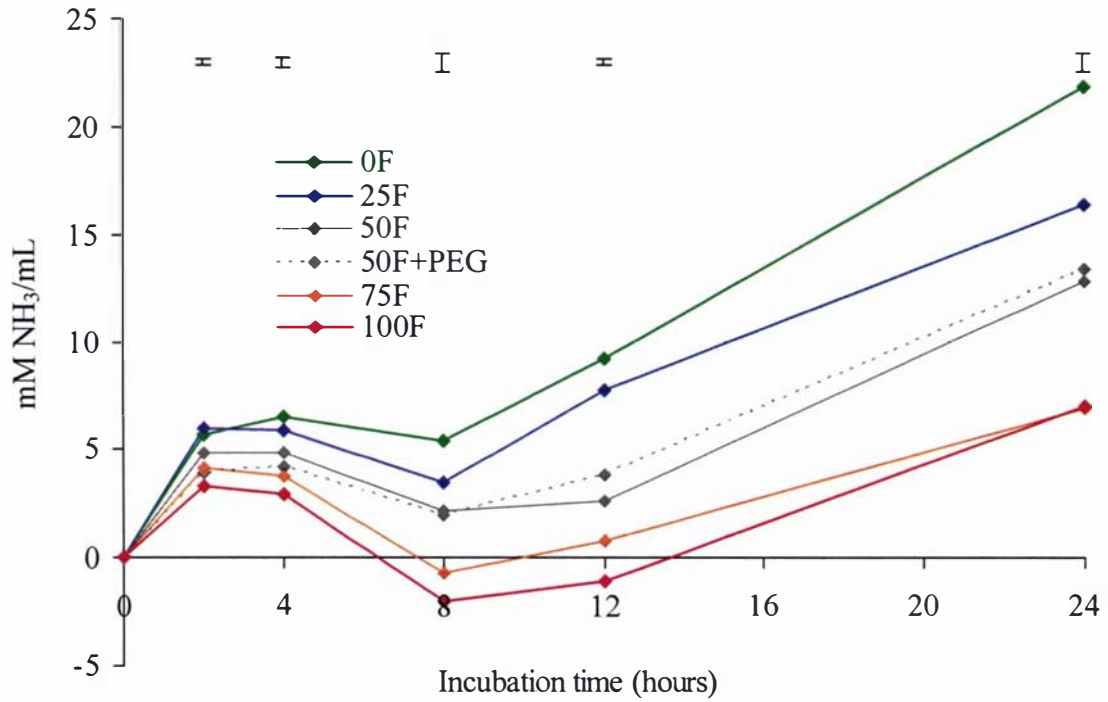


FIGURE 5.5 Net conversion of plant-N to ammonia-N from *in vitro* incubations of white clover herbage with different percentages of flower (F) and leaf, including one treatment containing PEG. Error bars represent SED's.

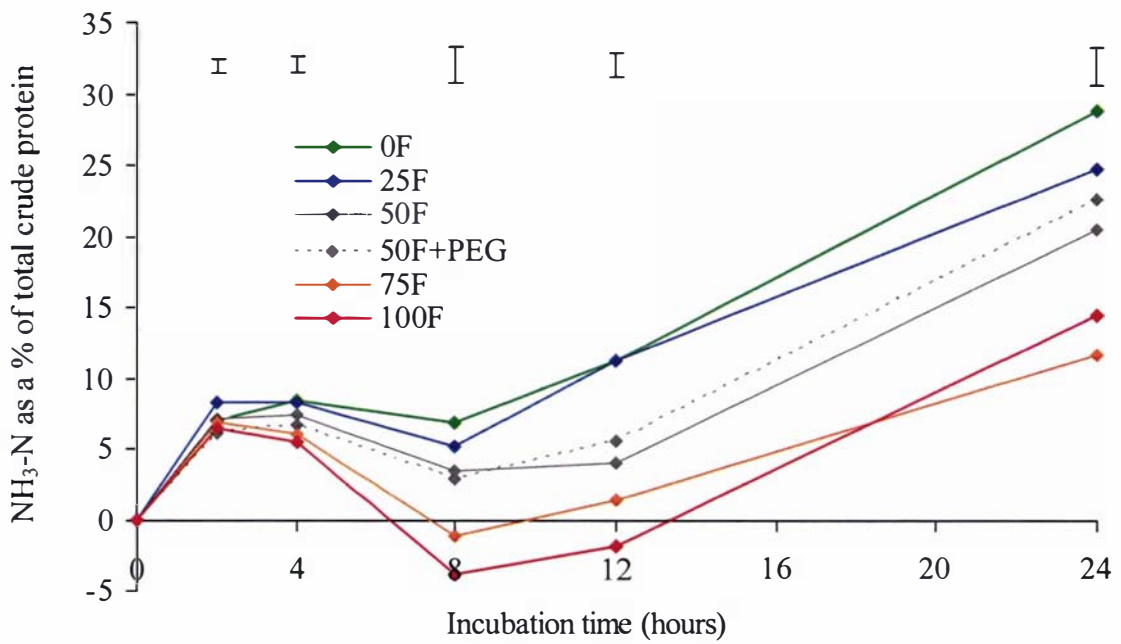


TABLE 5.5 Net rate of ammonia production (mM NH_3/g DM/h) during *in vitro* incubation of white clover herbage with different percentages of flower (F) and leaf, with PEG included in one treatment to remove the effects of condensed tannins.

Time	0F	25F	50F	50F+PEG	75F	100F	P linear	P PEG
0-2 h	0.095	0.103	0.081	0.069	0.068	0.057	***	*
2-4 h	0.018	0.000	0.002	0.006	-0.007	-0.009	**	NS
4-8 h	-0.010	-0.019	-0.022	-0.022	-0.036	-0.041	***	NS
8-12 h	0.030	0.037	0.003	0.015	0.013	0.009	**	NS
12-24 h	0.040	0.028	0.031	0.032	0.017	0.024	***	NS

TABLE 5.6 Net rate of conversion of plant N to ammonia N (%/h) during *in vitro* incubation of white clover herbage with different percentages of flower (F) and leaf, with PEG included in one treatment to remove the effects of condensed tannins.

Time	0F	25F	50F	50F+PEG	75F	100F	P linear	P PEG
0-2 h	3.49	4.12	3.59	3.07	3.38	3.23	*	*
2-4 h	0.67	0.01	0.09	0.29	-0.36	-0.51	**	NS
4-8 h	-0.04	-0.76	-0.99	-0.96	-1.81	-2.34	***	NS
8-12 h	1.10	1.50	0.15	0.66	0.62	0.49	*	NS
12-24 h	1.47	1.13	1.38	1.43	0.86	1.37	NS	NS

5.4.7 Volatile fatty acids

VFA production was rapid for the first 8 hours (Table 5.7), then slowed for the remainder of incubation. Total VFA concentrations were not affected by treatment for the first 12 hours of incubation (Figure 5.6), but at 24 hours, decreased linearly ($P < 0.05$) as flower contents increased, with a range of 4.6 to 5.4 mM VFA/g DM. The concentration of acetate followed the same trend as total VFA, with maximum rates of production (0.36-0.49 mM/g DM/h) in the first 2 hours (Table 5.7) and a maximum concentration of 3 mM/g DM at 24 hours. There were no significant treatment effects for acetate concentration or production rate (Table 5.7).

The rate of propionate production was most rapid between 2 and 4 hours of incubation (0.11-0.24 mM/g DM/h; Table 5.7). At 4 hours propionate concentrations were greater ($P < 0.05$) in 0F (0.65 mM/g DM) than in all other treatments (0.38-0.49 mM/g DM) (Figure 5.6). Propionate concentrations decreased linearly with increasing percentages of flower at each time (Figure 5.6). Butyrate production was fastest between 2 and 4 hours for 0F, and between 4 and 8 hours for all other treatments (Table 5.7). Increasing percentages of flowers caused a linear decrease in butyrate concentrations at each sampling time ($P < 0.001$; Figure 5.7).

The rate of minor VFA (valerate, isovalerate, isobutyrate) production was most rapid in the first 2 hours for all treatments except 0F (Table 5.7). Concentrations of minor VFA differed significantly between treatments after the first 2 hours, decreasing linearly with increasing percentages of clover flower (Figure 5.7; $P < 0.001$). At 8 hours the relationship between flower % and minor VFA concentration was quadratic ($P < 0.001$), with 0F having much greater concentrations than all other treatments.

The CT in the 50F treatment did not affect rates of total VFA production (Table 5.7) or acetate production (Table 5.7), but it reduced propionate concentrations by 12% at 12 h and 17% at 24 h ($P < 0.01$; Figure 5.6). CT also increased the rate of butyrate production by 22% ($P < 0.05$) between 4 and 8 hours (Table 5.7) so concentrations per unit DM were higher than in 50F+PEG at 8 ($P < 0.01$) and 12 hours ($P < 0.05$) (Figure 5.7). The CT reduced concentrations of minor VFA by 27% at 12 hours ($P < 0.05$), and by 16% at 24 hours in 50F relative to 50F+PEG (Figure 5.7).

The molar percentage of acetate was highest at 2 h of incubation for all treatments (70-80%), after which it declined and remained constant from 8 h onwards (Figure 5.8). This was mirrored by a rapid increase in the molar percentage of propionate (Figure 5.8), peaking at 4 h, and a slower increase with butyrate to maximum values at 8 h (Figure 5.9).

The molar percentages of all VFA were affected by treatment. Increasing flower contents resulted in linear increases in percentages of acetate and decreasing percentages of propionate, butyrate and minor VFA. The relationships between treatments were similar throughout the incubations (Figures 5.8 and 5.9). These trends were indicated by acetate to propionate (A:P) and acetate + butyrate:propionate (A+B:P) ratios (Figure 5.10) with highest values for 75F and 100F and lowest for 0F. At 4 h of incubation A:P ratios ranged from 2.3 to 3.5 (except when PEG was added to remove the CT effect of the 50F treatment).

Condensed tannins reduced the percentage of acetate at 4, 8 and 24 h relative to the 50F+PEG treatment, while increasing the percentage of propionate at 4 h ($P < 0.01$; Figure 5.8) and percentage of butyrate at all times (Fig 5.9). Addition of PEG to remove CT effects resulted in a high A:P ratio at 4 hours (4.4; $P < 0.001$). The A+B:P ratios followed a similar pattern to the A:P ratios over the incubation (Figure 5.10).

TABLE 5.7 Net rate of production (mM/g DM/h) of total VFA, acetate, propionate, butyrate and minor (isobutyrate, valerate and isovalerate) VFA during *in vitro* incubation of white clover herbage with different percentages of flower (F) and leaf, with PEG included in one treatment to remove the effects of condensed tannins.

VFA	Hour	0F	25F	50F	50F+PEG	75F	100F	P linear	P PEG
Total	0-2	0.51	0.67	0.57	0.61	0.61	0.53	NS	NS
	2-4	0.76	0.41	0.54	0.51	0.50	0.48	*	NS
	4-8	0.27	0.37	0.28	0.33	0.33	0.30	NS	NS
	8-12	0.13	0.15	0.14	0.22	0.12	0.20	NS	NS
	12-24	0.09	0.09	0.07	0.08	0.07	0.05	*	NS
Acetate	0-2	0.36	0.49	0.44	0.49	0.49	0.42	NS	NS
	2-4	0.40	0.21	0.30	0.34	0.29	0.31	NS	NS
	4-8	0.12	0.19	0.13	0.14	0.18	0.18	NS	NS
	8-12	0.07	0.09	0.07	0.13	0.10	0.13	NS	NS
	12-24	0.06	0.06	0.05	0.05	0.04	0.04	NS	NS
Propionate	0-2	0.089	0.107	0.076	0.076	0.072	0.073	*	NS
	2-4	0.236	0.136	0.171	0.114	0.150	0.135	*	*
	4-8	0.062	0.093	0.068	0.112	0.079	0.068	NS	*
	8-12	0.028	0.018	0.036	0.050	0.033	0.043	NS	NS
	12-24	0.010	0.022	0.007	0.013	0.011	-0.001	*	NS
Butyrate	0-2	0.052	0.053	0.039	0.032	0.038	0.033	***	NS
	2-4	0.094	0.055	0.057	0.055	0.045	0.039	***	NS
	4-8	0.078	0.090	0.077	0.063	0.065	0.051	***	*
	8-12	0.023	0.030	0.033	0.030	0.018	0.022	NS	NS
	12-24	0.012	0.008	0.010	0.011	0.007	0.009	NS	NS
Minor VFA	0-2	0.013	0.017	0.013	0.011	0.009	0.010	NS	NS
	2-4	0.026	0.012	0.010	0.003	0.010	0.000	**	NS
	4-8	0.013	0.007	0.006	0.013	0.005	0.008	[1]Quad*	NS
	8-12	0.006	0.017	0.004	0.006	0.007	0.005	[2]Cubic*	NS
	12-24	0.009	0.006	0.006	0.007	0.004	0.003	***	NS

[1] linear and cubic regressions were not significant, quadratic regression was.

[2]linear and quadratic regressions were not significant, cubic regression was.

FIGURE 5.6 Concentration of total VFA and propionate after 2, 4, 8, 12 and 24 hours from *in vitro* incubation of white clover herbage with different percentages of flower (F) and leaf, with PEG included in one treatment to remove the effects of condensed tannins. Error bars represent SED's.

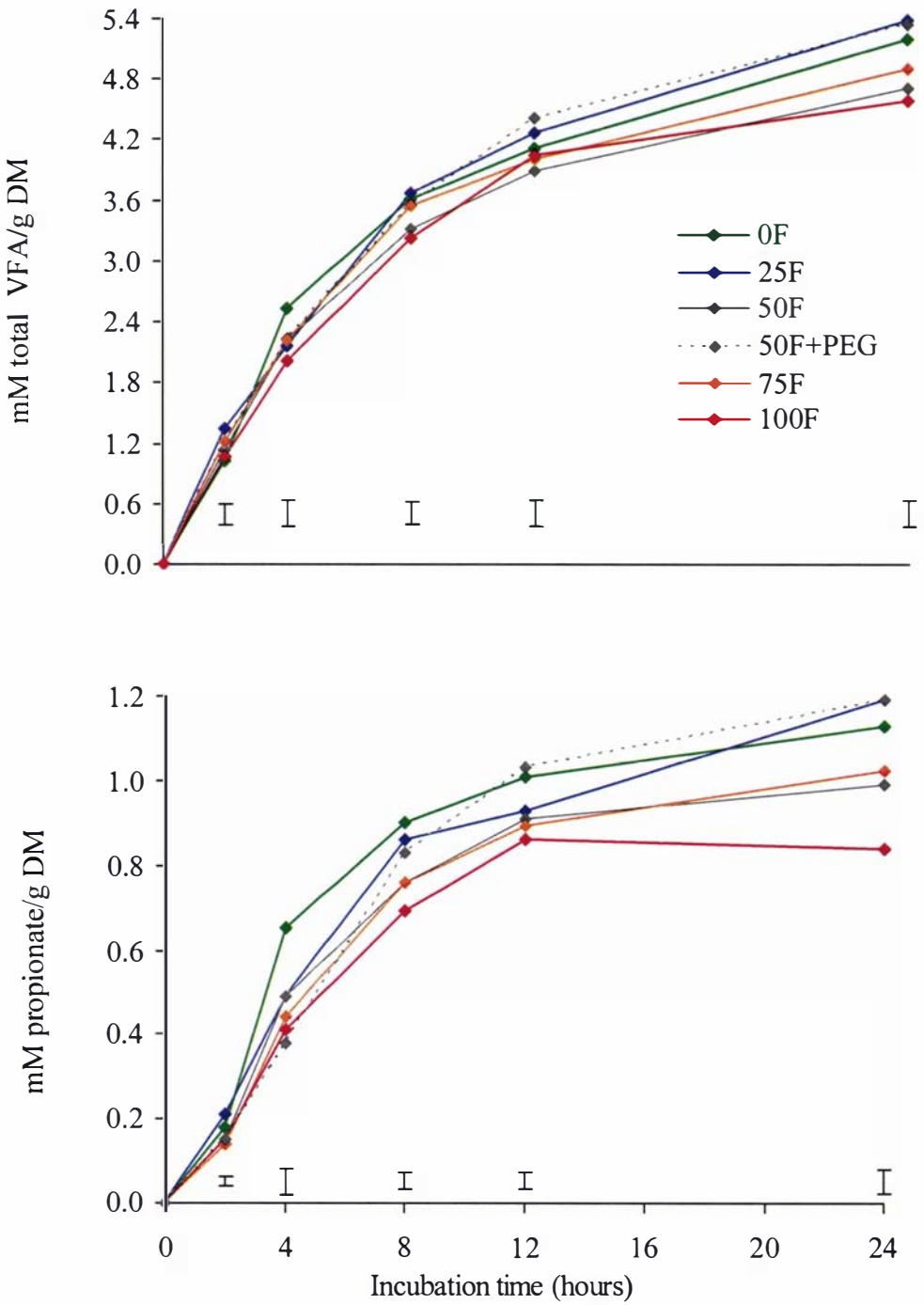


FIGURE 5.7 Concentration of butyrate and minor VFA (isobutyrate, valerate, isovalerate) after 2, 4, 8, 12 and 24 hours of *in vitro* incubation of white clover herbage with different percentages of flower (F) and leaf, with PEG included in one treatment to remove the effects of condensed tannins. Error bars represent SED's.

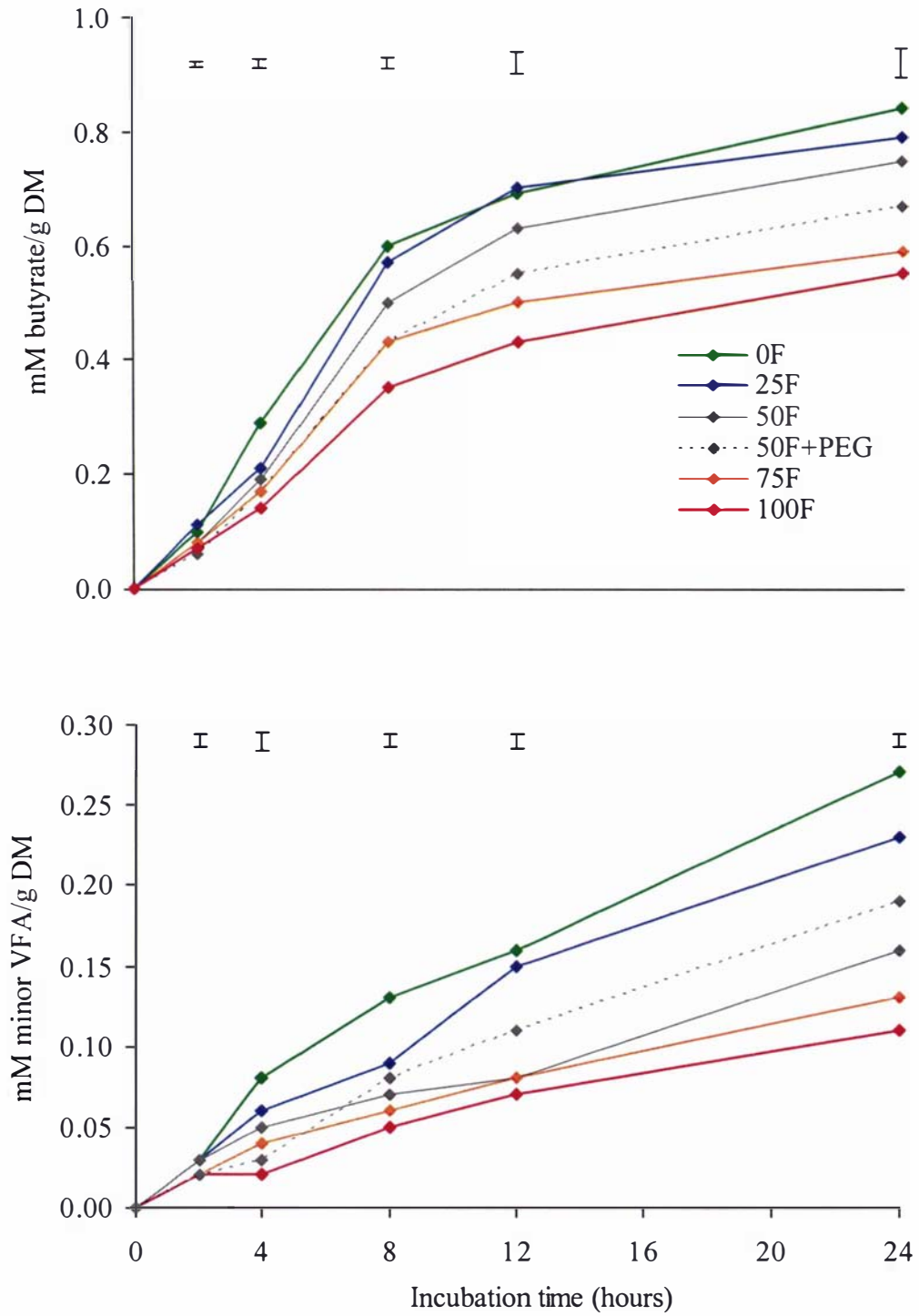


FIGURE 5.8 Molar percentage of VFA as acetate and propionate after 2, 4, 8, 12 and 24 hours of *in vitro* incubation of white clover herbage with different percentages of flower (F) and leaf, with PEG included in one treatment to remove the effects of condensed tannins. Error bars represent SED's.

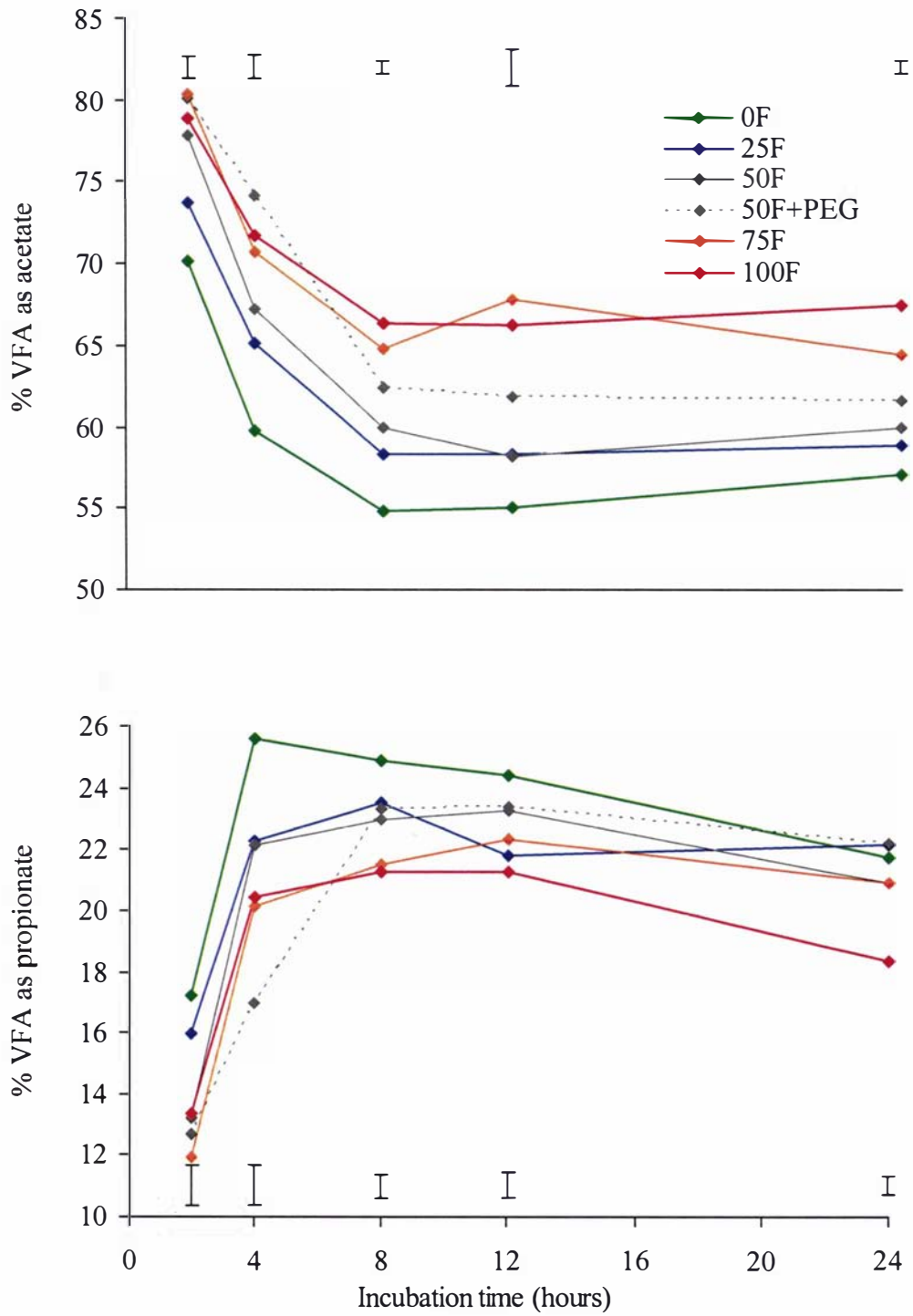


FIGURE 5.9 Molar percentage of VFA as butyrate and minor VFA (isobutyrate, valerate, isovalerate) after 2, 4, 8, 12 and 24 hours of *in vitro* incubation of white clover herbage with different percentages of flower (F) and leaf, with PEG included in one treatment to remove the effects of condensed tannins. Error bars represent SED's.

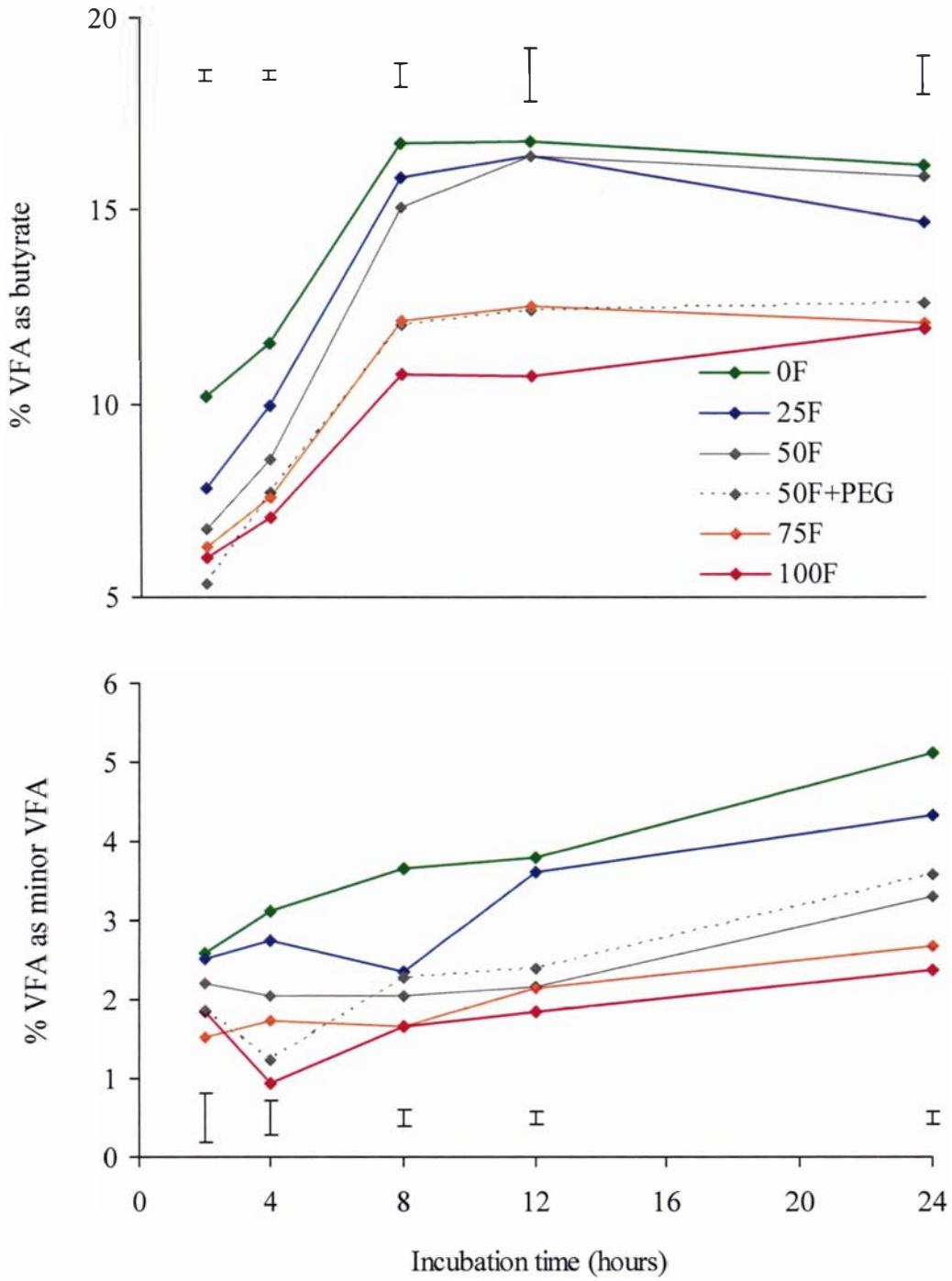
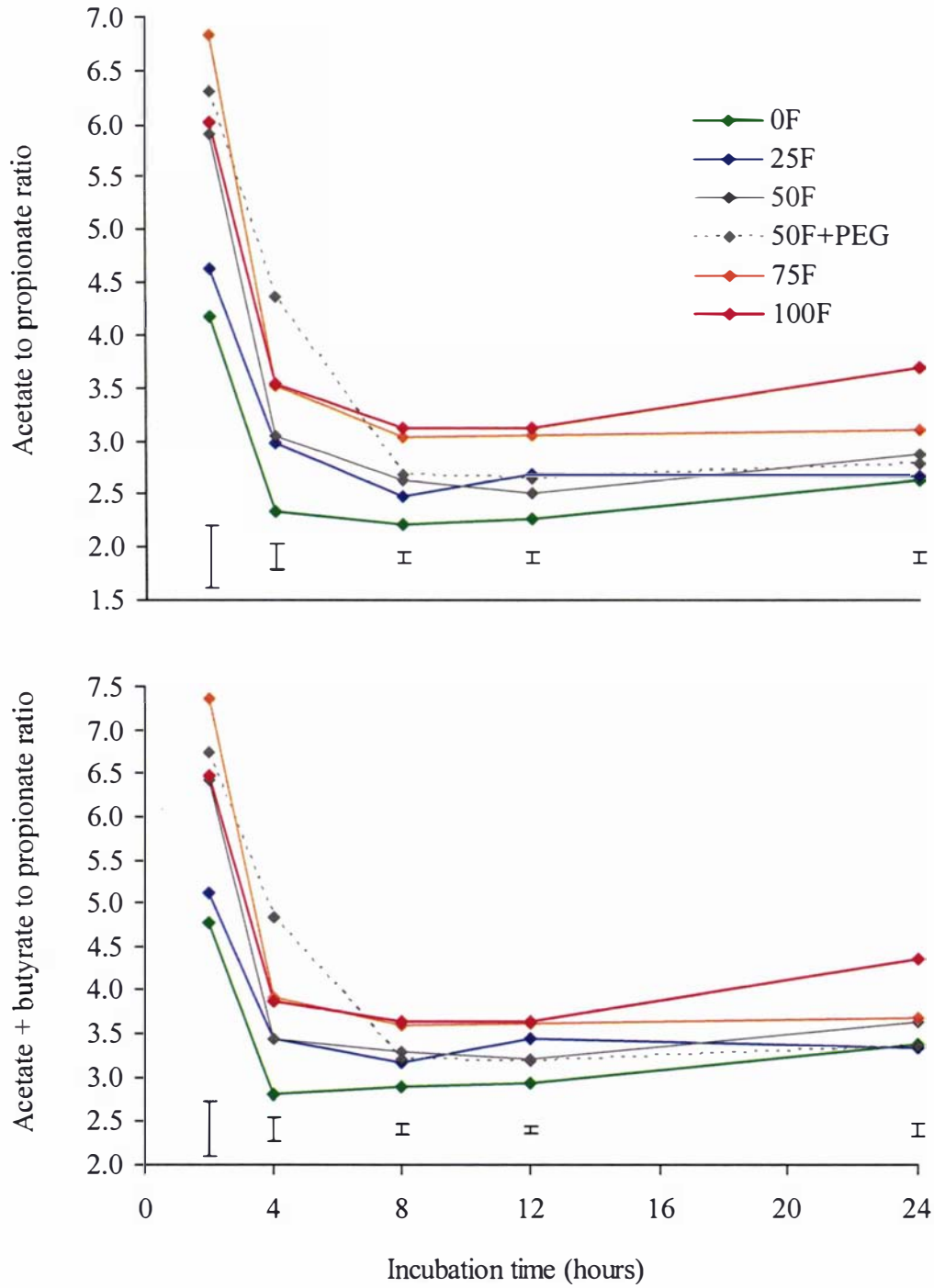


FIGURE 5.10 Ratio of acetate (A) to propionate (P) and A+ butyrate:P after 2, 4, 8, 12 and 24 hours of *in vitro* incubation of white clover herbage with different percentages of flower (F) and leaf, with PEG included in one treatment to remove the effects of condensed tannins. Error bars represent SED's.



5.5 DISCUSSION

Previous research has measured the digestion of white clover both *in vivo* (Cammell et al. 1986; Steg et al. 1994; Stockdale 1997; Cohen & Doyle 2001) and *in vitro* (Waghorn & Caradus 1994; Barrell et al. 2000; Burke 2004) with up to 25% of the clover DM as flowers. Research presented here has defined the effects of either white clover leaf or flower and combinations of each, as well as the effects of the CT contained within the flowers on the products of digestion. This information will assist white clover breeders in setting targets for CT production and flowering in future selections.

The extent of digestion of feed in the rumen is dependent on physical and microbial degradation. Digestion of white clover is rapid, with a rumen residence time of as little as 6 hours (Ulyatt 1969) and rumen organic matter digestibilities of 68 to 71% for cattle (Ulyatt et al. 1988). *In vitro* incubations in this experiment were conducted over 24 hours, but most emphasis is given to digestion in the first 12 hours of incubation, despite measurement of treatment differences at 24 hours incubation.

5.5.1 Feed analysis and particle size

The chemical composition of flowers and leaves were similar to previous reports, with flowers having lower crude protein concentrations and sometimes higher fibre concentrations (Gibb & Treacher 1983; Wilman & Altimimi 1984; Stockdale 1999). The availability of free CT (50% of total) from flowers indicates good potential for binding with leaf protein.

Mixing and mincing of herbage components before incubation was intended to mimic chewing, releasing cell contents and reducing the size of particles. Barrell et al. (2000) emphasised that the particle size distribution of minced forages (using similar equipment to this experiment) more closely resembled the boli of fresh forages swallowed by sheep and the rumen contents of cows fed fresh forages than does forage chopped into 6 mm lengths or freeze dried and ground. Particle sizes in this experiment were similar to those reported previously for minced white clover, white clover from sheep boli and the rumen contents of cows fed fresh lucerne (Table 5.8). The principal difference between minced and chewed forage was a lower proportion of minced material retained on the 4 mm sieve, and more on the 0.25 and 0.5 mm sieves.

The extent of forage particle breakdown during chewing and in the rumen depends on the forage type, rate of eating and the amount of chewing during grazing and rumination (Ulyatt et al. 1986). The particle size of clover flowers was greater than that for leaves, probably because some florets passed through the screen plate holes of the mincer without damage.

TABLE 5.8 Particle size distribution (% of DM retained on each sieve) from fresh white clover that has been minced while frozen to mimic chewing, and of swallowed boli from sheep fed white clover, or the rumen contents of cows fed lucerne (*Medicago sativa* L.) or perennial ryegrass (*Lolium perenne* L.).

Forage source	Sieve size (mm)[1]				Data souce
	4	1 + 2	0.25 + 0.5	<0.25	
Minced white clover[2]	1-9	17-28	27-35	35-40	This study
Minced white clover	8	33	21	38	Burke (2004)
Minced white clover	13	24	14	49	Waghorn & Caradus (1994)
Minced white clover	14	20	14	52	Barrell et al. (2000)
White clover boli swallowed by sheep	19	11	13	56	Waghorn & Shelton (1988)
Rumen contents of cows fed:					
Lucerne	16	18	23	43	Waghorn et al. (1989)
Perennial ryegrass	32	5	16	47	Waghorn et al. (1989)

[1] Length of square holes. 1 + 2 represents the sum of material retained on 1 and 2 mm sieves, 0.25 + 0.5 represents the sum of material retained on 0.25 and 0.5 mm sieves.

[2] Range across treatments.

5.5.2 Incubation medium pH

Incubations were buffered to minimise changes in pH arising from an accumulation of VFA in the media. Data in Chapter 4 indicate cows grazing white clover have a rumen pH range between 5.5 and 6.6, which is similar to *in vitro* values from 2 to 24 h (5.7 to 6.7). The rapid drop in pH in the first 12 hours of incubation implies rapid and extensive fermentation, with acidification by VFA exceeding buffering capacity.

5.5.3 Soluble protein concentration

Soluble protein is readily degraded by rumen bacteria and plant proteolytic enzymes (Kingston-Smith et al. 2003) unless it is protected by condensed tannins (McNabb et al. 1996). The concentration and disappearance during the incubation represents a balance between release due to mincing (at 0 h) and subsequent release from forage and its degradation by proteolytic enzymes.

Only 6.2% of white clover leaf crude protein was in the soluble fraction at 0 h, which was substantially less than *in sacco* measurements of chopped white clover (9-19% of CP; Cohen & Doyle 2001), minced white clover (38% of CP; Burke 2004) or swallowed white clover boli from cows (28-37%; Cohen & Doyle 2001). The difference between this and other studies is probably a function of measurement techniques. In the work presented here the soluble protein in the aqueous fraction of the media was truly dissolved, whereas *in sacco* studies determined soluble protein content by washing plant material in bags with 35 μm pore apertures. The 35 μm pores will enable organelles such as chloroplasts, which account for 75% of total leaf protein (Mangan 1982), to pass out of the bag. The lower proportion of soluble protein with increasing percentages of flower may indicate less rupture of flower cells (corresponding to a higher proportion of flower on larger sieve sizes).

The CT did not affect the percentage of soluble protein (Table 5.3) at 0 h, possibly because the pH of the medium (7.1) was at the upper limit for stable CT-protein complexes. Jones & Mangan (1977) reported maximum stability between pH 3.5 and 7.0 and the brief period between thawing and sampling (about 90 minutes) may have limited stable complex formation (Diaz-Hernandez et al. 1997). However, addition of PEG would have minimised effects of CT on digestion after the pH declined (2 hours). CT did reduce soluble protein concentration at 2 and 4 h of incubation. The effects of

increasing percentages of flowers, and the high proportion of unbound (free) CT in white clover flowers complement the tendency for lower soluble protein concentrations in cows grazing high tannin (HT) white clover compared to Huia white clover (Chapter 4).

5.5.4 Proteolysis

A high concentration of ammonia and low concentrations of 'minor' VFA are indicators of protein degradation, but interpretation is affected by ammonia utilisation for microbial growth and the crude protein concentration of the plant material. Replacement of leaf by flower lowered the CP content of the DM and increased the CT concentration. Soluble protein is the most extensively and rapidly degraded protein fraction (Chalupa 1984). At 24 h, 29% of clover leaf CP was recovered as ammonia, which is similar to reports of 21-40% net degradation of white clover leaves by Caradus et al. (1995), but lower than the 49% at 24 h by Barrell et al. (2000) and Burke (2004).

Increasing the percentage of white clover as flowers reduced initial soluble protein concentrations, ammonia concentrations and the percentage of plant N converted to ammonia. Satter & Slyter (1974) suggested a minimum ammonia concentration of 50 mg/L (3.6 mM/L) to maximise bacterial growth. When clover flowers comprised 75% or more of DM bacterial growth may have been limited at 8 to 12 h, however, such high flower contents are unlikely to occur in pastures.

The CT in the 50F treatment did not reduce ammonia or minor VFA production (Tables 5.6 and 5.7), suggesting a minor impact on protein digestion. Although the CT concentration (26 g/kg DM) was low relative to the concentration of CP, the structure (100% prodelphinidin; Jones et al. 1976) suggested a good potential for inhibiting proteolysis. Prodelphinidins are more inhibitory to proteolysis than the procyanidin component that dominates in birdsfoot trefoil (*Lotus corniculatus* L.) CT (McNabb et al. 1997; Aerts et al. 1999). CT are able to reduce proteolytic enzyme activity (Barry & Manley 1986) and proteolytic bacterial populations (Molan et al. 2001; Min et al. 2002), although these effects depend on the type of CT (Nelson et al. 1997). CT-protein complexes also reduce the susceptibility of forage protein to microbial degradation (Min et al. 2003).

5.5.5 Volatile fatty acids

The percentage of clover flower or CT had minor effects on rates of VFA production (Table 5.7). Increasing the percentage of flower in incubations increased the percentage of VFA as acetate and reduced propionate yields and A:P ratios. This will lower the gluceogenic potential (Holmes et al. 2002b), which could reduce milk lactose synthesis; the driver of milk yield (Holmes et al. 2002c). However, the low NDF concentrations in leaves (240 g/kg DM) and flowers (258 g/kg DM) would favour a low A:P ratio.

Trials evaluating CT effects in sheep fed birdsfoot trefoil or lotus major have not demonstrated effects on VFA molar percentages (except minor VFA) (Waghorn et al. 1987b, 1994b). *In vitro* incubations have demonstrated negative effects of CT on the yields of propionate and isobutyrate (McMahon et al. 1997) but effects on total VFA concentrations were small. In this experiment, white clover CT increased the molar percentage of butyrate and Terrill et al. (1992b) reported increased percentages of butyrate in sheep rumen fluid due to the CT in sulla (*Hedysarium coronarium* L.). They suggested the CT in sulla increased rumen protozoal populations, which are able to produce butyrate (van Soest 1982; McNabb et al. 1989). High concentrations (>50 g CT/kg DM; Barry et al. 1986; Waghorn et al. 1994a) or highly astringent CT (eg in erect dorycnium (*Dorycnium rectum* L.) or carob (*Ceratonia silique* L.)) are able to reduce bacterial digestion *in vivo* (Priolo et al. 2000; Waghorn & Molan 2001) but the CT in white clover flowers (52 g/kg DM) did not limit total VFA production *in vitro*.

5.6 CONCLUSIONS

Increasing the percentage of white clover present as flowers in *in vitro* incubations reduced the degradation of plant protein to ammonia, partly due to reduced solubility of the protein, but the CT in the flowers had minimal impact on proteolysis. When flowers comprised 75% or more of clover DM ammonia concentrations may have limited microbial growth, possibly due to a slow release of protein from plant cells. Treatments did not affect total VFA production, but the percentage of VFA as acetate increased, while that of propionate and butyrate decreased with increasing flower contents. The CT in the flowers increased butyrate production.

CHAPTER 6

In vitro digestion of perennial ryegrass leaf with white clover flowers or birdsfoot trefoil

CHAPTER 6: *In vitro* digestion of perennial ryegrass leaf with white clover flowers or birdsfoot trefoil

6.1 ABSTRACT

This experiment determined the effects of including white clover (*Trifolium repens* L.) flowers (F) or birdsfoot trefoil herbage (BT; *Lotus corniculatus* L.) as a source of condensed tannins (CT) with ryegrass on rumen proteolysis and VFA production *in vitro*. Incubations with buffered rumen fluid comprised minced perennial ryegrass (*Lolium perenne* L.) leaf (100R) and mixtures of white clover flowers (F; 16, 33 and 50% of dry matter (DM)) with ryegrass leaf and mixtures of birdsfoot trefoil (50% of DM) with ryegrass leaf (50BT), with and without polyethylene glycol (PEG). CT concentrations in the 16, 33 and 50% F treatments were 8.4, 17.3 and 26.2 g/kg DM respectively, and 15.7 g/kg DM for 50BT. White clover flowers contained 155 g crude protein/kg DM, compared with 253 g/kg in ryegrass leaves and 285 g/kg in BT. Clover flowers were more effective at reducing soluble protein concentrations from 0 to 4 hours of incubation than was birdsfoot trefoil (average of 271 versus 385 mg/L), due partly to the lower protein content of the flowers. The percentage of plant N present as ammonia-N at 24 hours of incubation decreased with increasing percentages of clover flowers ($P < 0.001$), from 27% for 100R to 13% for 50F. With 50BT, 24% of N appeared as ammonia-N at 24 hours of incubation, and removing the CT effect with PEG increased this to 27%. Treatment effects on total VFA concentration were minor, but birdsfoot trefoil treatments had the lowest acetate to propionate ratios, partly due to the CT. Although white clover flowers reduced ruminal protein degradation in ryegrass-based diets, this appeared to be a result of lower plant N concentrations and possibly less cell rupture, rather than the effects of floral CT. A high proportion of white clover flowers in ryegrass based pastures is unlikely to improve amino acid absorption or propionate availability for dairy cow performance.

6.2 INTRODUCTION

New Zealand agriculture is based predominantly on mixed swards of perennial ryegrass and white clover. Green leaf is the highest quality component of perennial ryegrass, and is selectively grazed in preference to stem, flower and dead material by cattle (Forbes & Hodgson 1985) and sheep (Rattray & Clark 1984). Both ryegrass leaf and white clover crude protein (CP) concentrations often exceed ruminant requirements for high levels of production. The protein is rapidly degraded by rumen microbes, often in excess of their requirements (Burke et al. 2000; Chaves et al. 2002).

Condensed tannins (CT) protect plant proteins from microbial degradation in the rumen, reducing the conversion of protein to ammonia. White clover flowers contain CT (Jones et al. 1976), and a high proportion of DM as flowers in a pure clover diet can reduce rumen ammonia concentrations both *in vivo* (Chapter 4) and *in vitro* (Chapter 5). CT from one plant species can also bind with and precipitate protein from another species both *in vitro* and *in vivo* (Waghorn & Jones 1989; Min et al. 2000). This is most likely to occur when the concentration of unbound CT in a plant exceeds that required to bind all of its own protein (Barry & McNabb 1999).

The CT from white clover flowers may bind with and protect ryegrass protein from microbial degradation, depending on the percentage of clover flowers in the diet. The optimum clover content in a pasture diet for maximising milk production is between 50 and 65% of DM (Harris et al. 1997), however, white clover normally makes up only 15 to 20% of DM in New Zealand dairy pastures. This experiment determined the effects of white clover flowers on ryegrass digestion *in vitro*, when mixtures of 0, 16, 33 and 50% white clover flowers (on a DM basis) were incubated with perennial ryegrass leaf.

The CT in birdsfoot trefoil reduce protein degradation in the rumen, increase amino acid supply to the small intestine (Waghorn et al. 1987b), and can increase milk production by 10 to 20% when fed to dairy cows (Woodward et al. 1999; 2000; 2004). Birdsfoot trefoil was incubated with ryegrass leaf in this experiment to compare its *in vitro* digestion and the effects of its CT with that from white clover flowers. One birdsfoot trefoil treatment included PEG, which preferentially binds with CT (Jones & Mangan 1977), to differentiate between the effects of CT and other aspects of forage composition.

6.3 MATERIALS AND METHODS

6.3.1 Experimental design

In vitro incubations of the following treatments were performed using the methods described in Chapter 5. The mixtures represent a range of legume contents up to the optimal clover content for milk production (50%). Forages were perennial ryegrass leaf (cultivar Bronsyn), HT white clover (see Section 2.4.4) flowers, and birdsfoot trefoil (cultivar Grasslands Goldie) herbage. All incubations were carried out simultaneously.

1. 100% ryegrass leaf (100R)
2. 84% ryegrass leaf; 16% flower (16F)
3. 67% ryegrass leaf; 33% flower (33F)
4. 50% ryegrass leaf; 50% flower (50F)
5. 50% ryegrass leaf; 50% birdsfoot trefoil (50BT)
6. 50% ryegrass leaf: 50% birdsfoot trefoil + PEG (50BT+PEG)

Triplicate bottles of each treatment were removed from the incubator after 0, 2, 4, 8, 12 and 24 hours of incubation.

6.3.2 Collection and preparation of forage mixtures and measurements

Forage samples were harvested with hand shears between 1 and 2 pm on 28 January 2003 and were immediately placed in a plastic bag on ice, then transferred to a -18°C freezer. Approximately 600 g of birdsfoot trefoil herbage (leaf plus stem) and 800 g of perennial ryegrass leaf blades were harvested. White clover flowers were sampled as described in Chapter 5. Birdsfoot trefoil pastures were 15 cm long and herbage was cut to 5 cm above ground level. Perennial ryegrass was 15 to 20 cm long, and was cut to 5 to 8 cm above ground level to avoid stem and dead material. Any ryegrass stem or dead material inadvertently collected was discarded.

Two days after collection, frozen herbage samples were chopped into 2-3 cm lengths (Plates 6.1 and 6.2), for ease of mincing and returned to the freezer once chopping was complete. Samples were sub-sampled for DM% determination, mixed to form the respective treatments then minced and incubated with buffer, reducing agent and

inoculum using methods and cows fed perennial ryegrass/white clover pastures as described in Chapter 5.

Chemical analysis of forages, particle size determination of minced material, incubation procedures and measurements are those described in Section 5.3. Minced samples of clover flower (100F) and 50BT were freeze-dried, then analysed by the butanol-HCl colorimetric technique of Terrill et al. (1992a) for CT determination. The composition of mixtures was calculated from the proportions of individual components.

Upon removal from the incubator, pH was measured and samples (including rumen fluid inoculum) were sub-sampled for analysis of soluble protein, VFA (excluding 2 hour samples) and ammonia concentration. The 2 hour incubation samples were not analysed for VFA concentration to reduce analysis costs.

6.3.3 Calculation of data and statistical analyses

In vitro concentrations of NH_3, VFA and soluble protein were adjusted to remove contributions from the rumen inocula, as described in Section 5.3.7. VFA data are expressed in mM per gram of forage DM incubated. Ammonia data are expressed as mM/L and as a percentage of plant N incubated. Soluble protein concentrations are presented in mg/L and as a percentage of the crude protein incubated for each bottle.

All data were subjected to analysis of variance with Genstat 5, using the model for completely randomised experimental designs. SED's accounted for the variation in the 0 hour samples as well as the variation between replicates at each time period. Differences were treated as significant at $P < 0.05$. Data were analysed for differences among treatments for each incubation period, differences among incubation times, and interactions between treatments and incubation times. Linear, quadratic and cubic regression analysis was performed using Genstat 5 to determine responses to increasing percentages of clover flower relative to ryegrass leaf at each incubation time.

PLATE 6.1 Frozen chopped perennial ryegrass leaf, ready for mincing.

PLATE 6.2 Frozen, chopped birdsfoot trefoil ready for mincing.

6.4 RESULTS

6.4.1 Feed composition

Clover flowers had a lower NDF and crude protein concentration in the DM than ryegrass leaves (Table 6.1) but a higher DM% and soluble carbohydrate concentration. Birdsfoot trefoil had the lowest DM%, NDF and ADF concentration, but the highest CP, soluble carbohydrate and lipid concentration (Table 6.1). 50F had a higher DM% CT and NDF concentration, but lower CP concentration than 50BT.

Minced clover flowers (100F) contained 52.4 g CT/kg DM, of which 50% was free (unbound), 44% was protein bound and 6% was fibre bound. The minced mixture of birdsfoot trefoil and ryegrass (50BT) contained 15.7 g CT/kg DM, of which only 8% was free, 75% was protein bound, and 17% was fibre bound.

TABLE 6.1 Dry matter percentage (DM%) at harvest, and chemical composition (g/kg DM) of individual species and mixtures of perennial ryegrass leaf (R) with white clover flower (F) or birdsfoot trefoil (BT). Data are from one sample of F, BT and R.

Treatment	DM%	CP	CHO	NDF	ADF	Lipid	CT
100F[a]	19.6	155	149	258	256	32	52.4
100BT[a]	16.4	285	179	191	146	42	31.4
100R	18.8	253	108	395	194	36	0.0
16F	18.9	237	115	373	204	35	8.4
33F	19.1	221	122	350	214	35	17.3
50F	19.2	204	129	327	225	34	26.2
50BT	17.6	269	144	293	170	39	15.7

[a] Not a treatment, 100F = 100% white clover flowers, 100BT = 100% birdsfoot trefoil. For treatment descriptions see 6.3.1.

CP = crude protein, CHO = soluble carbohydrates, NDF = neutral detergent fibre, ADF = acid detergent fibre, CT = condensed tannins.

Less than 50% of DM was retained on the 4 mm sieve for all treatments (Table 6.2). An average of 15% was retained on the 1 and 2 mm sieves, 23% on the 0.25 and 0.5 mm sieves, and 35% of DM was smaller than 0.25 mm. 50BT produced the smallest particles with the highest proportion of soluble DM.

TABLE 6.2 Particle size distribution (% distribution in dry matter) of minced ryegrass leaf (R) mixed with different percentages of either white clover flower (F), or birdsfoot trefoil (BT). Data are from one sample per treatment.

Treatment	Particle size						
	> 4 mm	2-4 mm	1-2 mm	0.5-1.0 mm	0.25-0.50 mm	residues	soluble
100R	40.7	4.3	9.7	7.8	16.5	10.4	10.6
16F	45.0	4.5	7.7	7.0	10.8	10.2	14.9
33F	8.8	10.3	12.1	10.9	17.3	13.0	27.6
50F	33.2	3.0	7.6	5.4	13.0	9.2	28.8
50BT	4.5	6.9	9.6	7.2	19.7	16.0	36.1

6.4.2 Inocula and 0 hour incubation samples

The pH at 0 hours (h) decreased with increasing percentages of clover flower ($P < 0.001$) (Table 6.3, Figure 6.1). Soluble protein concentrations were highest in the 100R and birdsfoot trefoil treatments (507-619 mg/L), and ranged from 351 to 391 mg/L in treatments containing clover flowers, which was similar to that in the rumen inocula. 100R had lower ammonia concentrations than treatments with clover flowers or birdsfoot trefoil (Table 6.3). VFA concentrations at 0 h were lowest in 100R (111 mM/L), intermediate in treatments with clover flowers (132-137 mM/L), and highest in treatments with birdsfoot trefoil (156 and 158 mM/L).

TABLE 6.3 Characteristics of the rumen fluid (inocula) used for incubations and comparable concentrations in incubation bottles at 0 hours for each treatment. Concentrations for each treatment are adjusted for dilution of rumen fluid by buffer and reducing agent, and assuming 50% of the wet weight of forage mixtures had been released as water from plant cells[1].

Parameter	Inocula	100R	16F	33F	50F	50BT	50BT+PEG
pH (non-adjusted)	6.26	7.25	7.14	7.13	7.10	7.05	7.05
Ammonia (mM/L)	16.7	15.0	18.2	18.8	19.1	18.3	18.8
Soluble protein (mg/L) (non adjusted)	363	603	358	391	351	507	619
Total VFA (mM/L)	116	111	132	137	133	156	158
Acetate (mM/L)	78.6	73.6	88.9	93.2	90.3	106.9	95.2
Propionate (mM/L)	21.7	19.4	22.8	23.3	22.7	26.0	22.6
Butyrate (mM/L)	14.6	12.8	14.5	14.7	14.4	16.5	14.5
Minor VFA[2] (mM/ L)	5.3	4.9	2.5	3.4	5.6	6.5	6.0

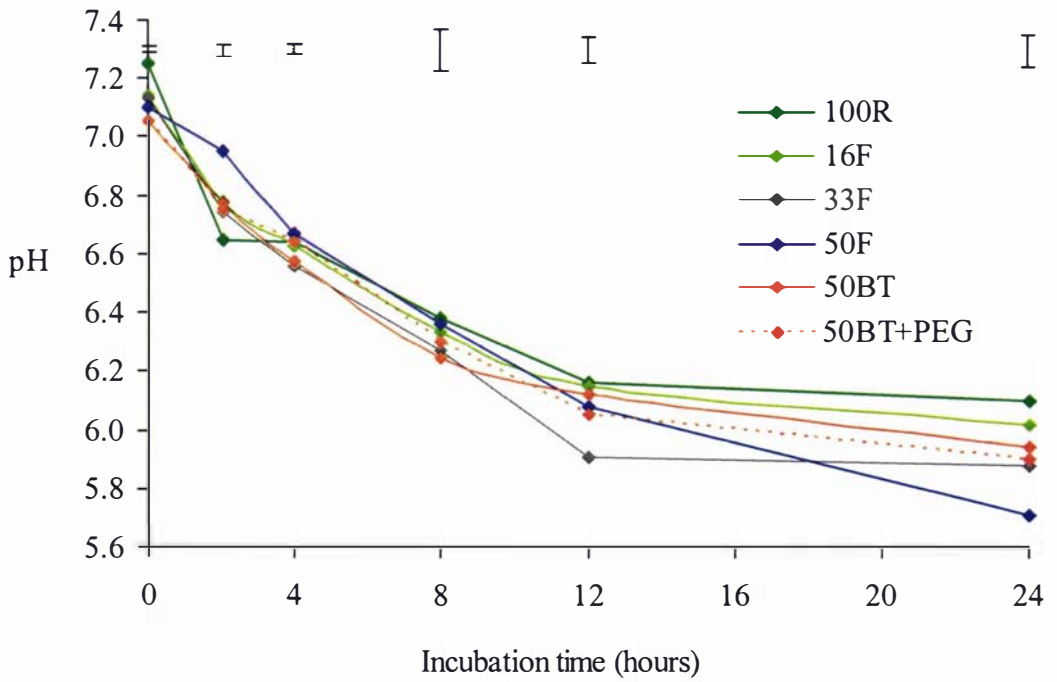
[1] Chewed forage is similar to minced forage, with about 60% of cells broken by chewing during eating (Waghorn et al. 1989). Given that equilibration between cell contents (from a ruptured cell) and the buffered inocula will take some time, an initial value of 50% is used for the amount of water released from cells into the incubation medium.

[2] Minor VFA = isobutyrate, valerate, isovalerate

6.4.3 Incubation medium pH

Initial pH (7.05-7.25) was highest in the 100R treatment and declined rapidly in the first 2 hours of incubation (Figure 6.1). The pH of all treatments declined between 0 and 12 hours incubation with little change thereafter, except in 50F. At 24 hours there was a negative linear relationship between pH and % flower ($P < 0.01$). 50BT was similar to 50BT+PEG throughout the incubation.

Figure 6.1 Change in pH during *in vitro* incubations of perennial ryegrass leaf (R) with different percentages of white clover flower (F) or birdsfoot trefoil (BT) with and without PEG. Error bars represent SED's.



6.4.4 Soluble protein

The concentration of soluble protein was always higher in ryegrass alone (100R) compared to ryegrass plus clover flowers (Figure 6.2). Clover flowers reduced soluble protein concentrations by 18 to 47% compared to 100R, but there was little difference between the flower treatments (16F, 33F, 50F) over the first 12 hours of incubation. Whilst the soluble protein concentration in 100R declined over the first 4 hours of incubation and increased from 12 to 24 h, the values for incubations containing flowers were relatively constant over the entire incubation period.

Treatments containing BT also had higher soluble protein concentrations compared to those with white clover flowers over the first 4 h of incubation (Figure 6.2). The CT in birdsfoot trefoil decreased soluble protein concentrations by 20% at 0 h ($P < 0.01$) and 15% at 4 h ($P < 0.01$) (Figure 6.2) but overall effects of CT in the 50BT treatments were minor.

The soluble protein as a percentage of plant crude protein incubated was consistently less than 7.5% in all treatments (Figure 6.3). Differences between 100R and treatments containing clover flowers were only evident at 0, 2 and 24 h. At 2 h, 50BT had a greater percentage of its crude protein as soluble protein than 50F, with the reverse effect at 8, 12 and 24 hours ($P < 0.001$).

Figure 6.2 Soluble protein concentration of plant origin in the medium from *in vitro* incubations of perennial ryegrass leaf (R) with different percentages of white clover flower (F) or birdsfoot trefoil (BT) with and without PEG. Error bars represent SED's.

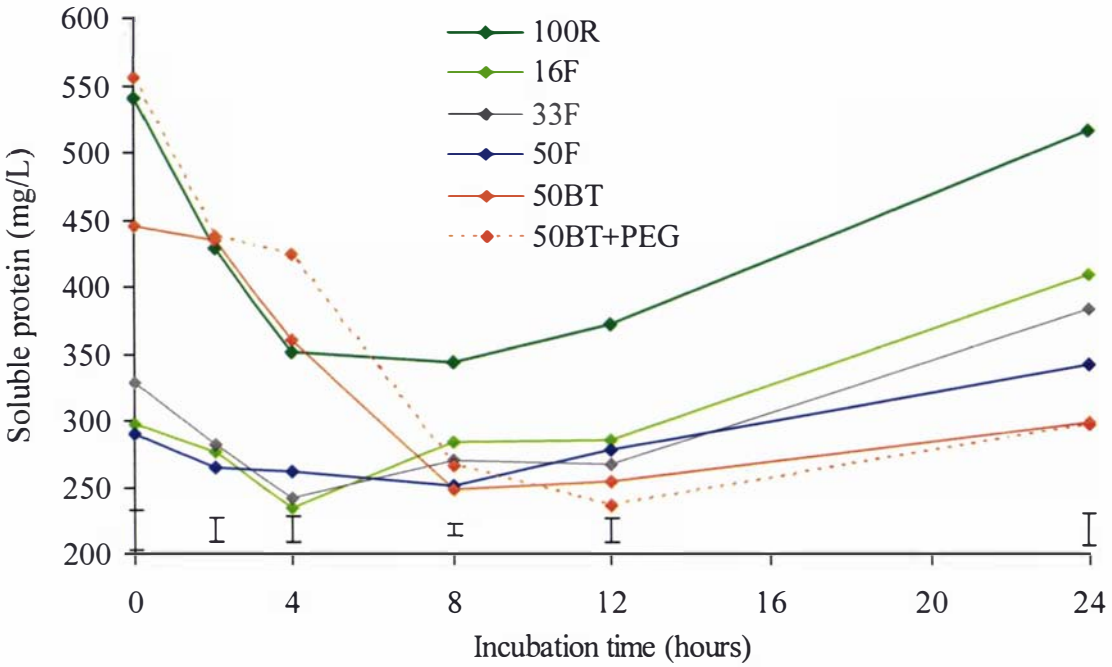
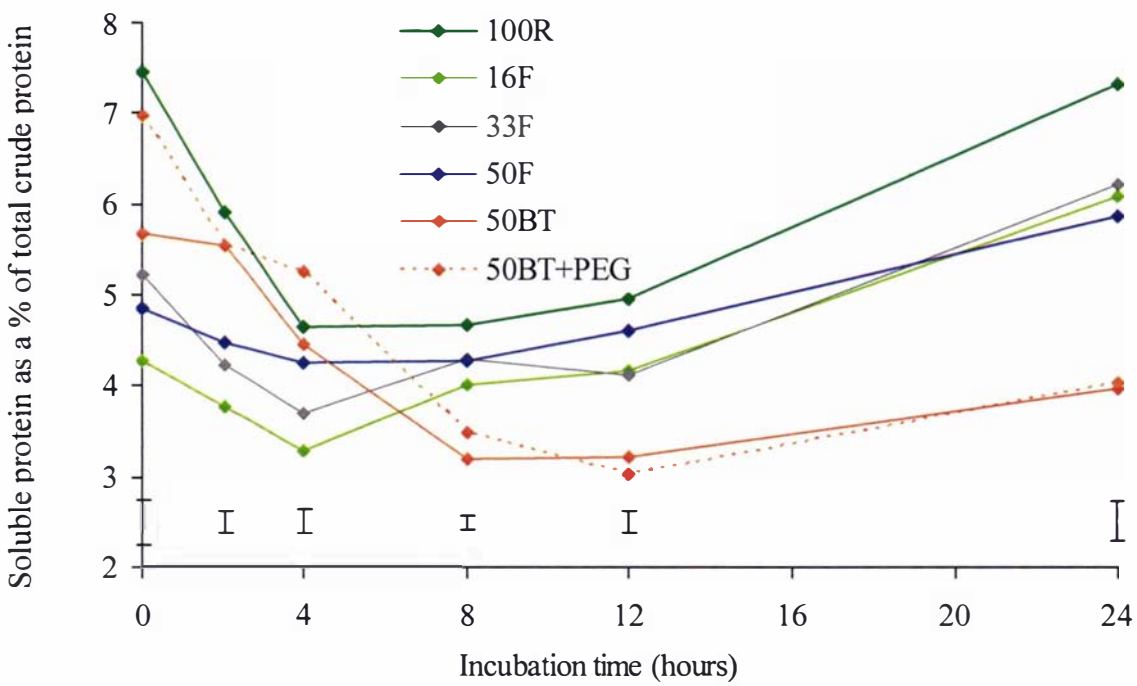


Figure 6.3 Soluble protein as a percentage of plant protein from *in vitro* incubations of perennial ryegrass leaf (R) with different percentages of white clover flower (F) or birdsfoot trefoil (BT) with and without PEG. Error bars represent SED's.



6.4.5 Ammonia

The change in ammonia concentration over time followed a similar pattern for all treatments (Figure 6.4). The pattern of ammonia expressed as a percentage of plant N as NH_3-N (Figure 6.5) was also similar for all treatments, with apparent conversion of plant N to NH_3-N most rapid in the first 2 h of incubation (Table 6.4). NH_3 concentrations dropped between 4 and 8 hours of incubation for treatments containing clover flowers, but always remained above 0 h values. The % plant N appearing as NH_3-N decreased linearly ($P < 0.001$) with increasing flower contents at all times, with the largest treatment differences at 24 hours (range of 13.1 (50F) to 26.7% (100R)).

50BT had higher ammonia concentrations than 50F from 4 (4.9 versus 3.5 mM/L, respectively, $P < 0.001$) to 24 hours (20.4 versus 8.9 mM/L, respectively, $P < 0.001$) (Figure 6.4). A higher percentage of 50BT plant N appeared as NH_3-N compared to that from 50F from 8 (6.0 versus 1.7, respectively, $P < 0.001$) to 24 hours (23.7 versus 13.1%, respectively, $P < 0.001$) (Figure 6.5).

Maximum NH_3 concentrations occurred at 24 hours for all treatments, when 50BT+PEG had the same percentage of plant N present as NH_3-N as 100R (26.7%). Birdsfoot trefoil CT only affected ammonia concentration at 24 hours, causing a 9% reduction ($P < 0.05$) (Figure 6.4).

FIGURE 6.4 Net ammonia concentration from *in vitro* incubations of perennial ryegrass leaf (R) with different percentages of white clover flower (F) or birdsfoot trefoil (BT) with and without PEG. Error bars represent SED's.

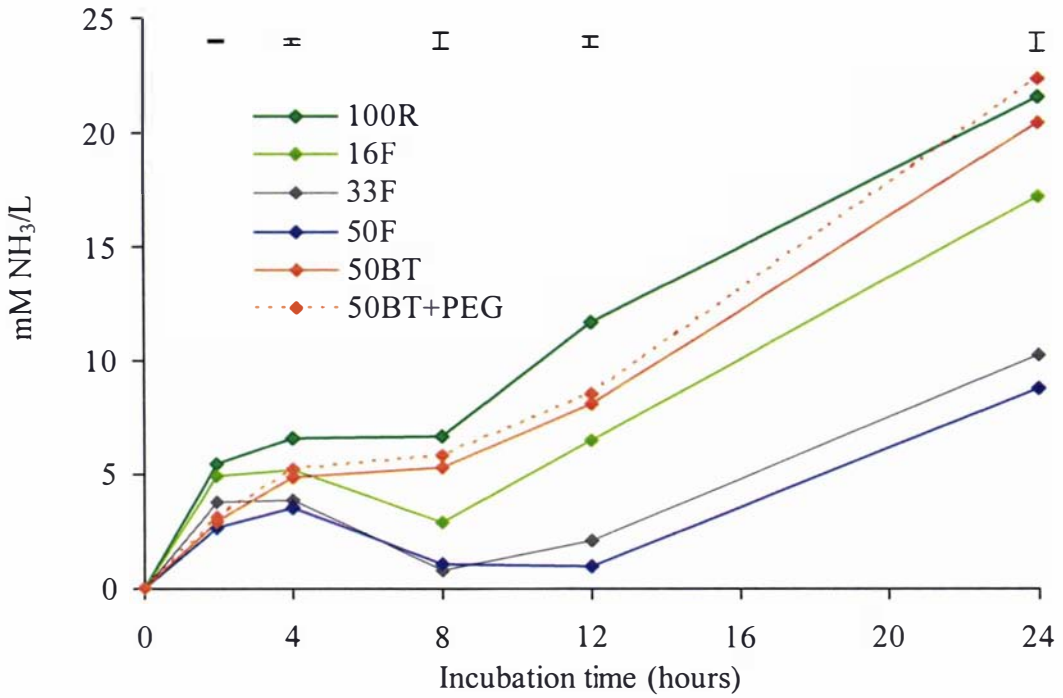


FIGURE 6.5 Net conversion of plant N to ammonia-N from *in vitro* incubations of perennial ryegrass leaf (R) with different percentages of white clover flower (F) or birdsfoot trefoil (BT) with and without PEG. Error bars represent SED's.

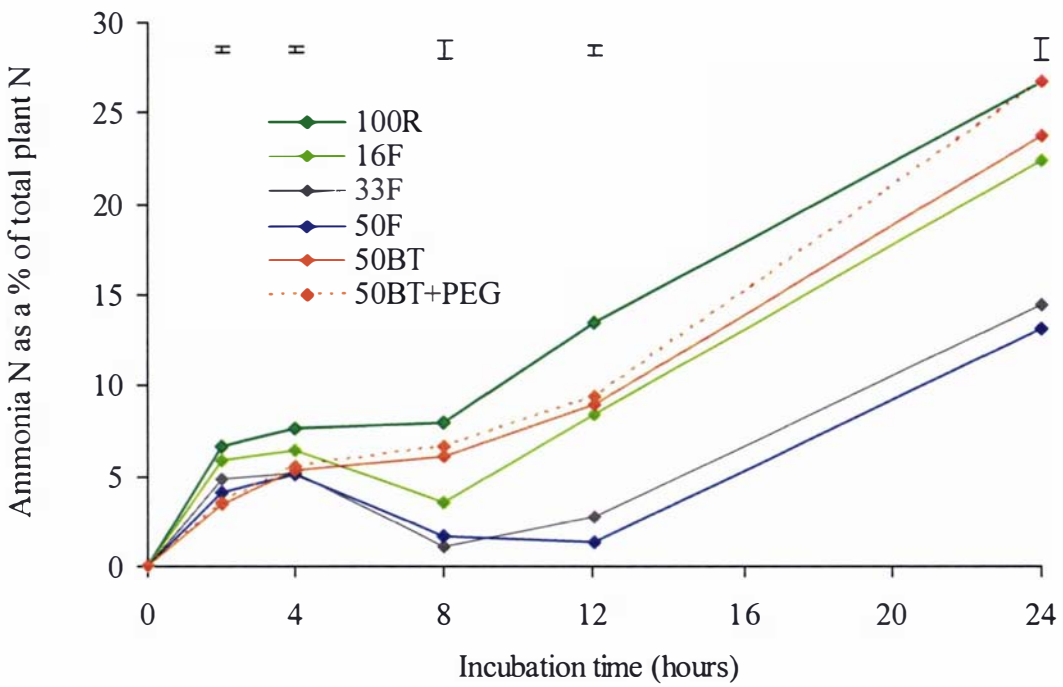


TABLE 6.4 Net rate of conversion of plant N to NH_3-N (μM NH_3/mM plant N/h) from *in vitro* incubations of perennial ryegrass leaf (R) with different percentages of white clover flowers (F) or birdsfoot trefoil (BT) with and without PEG.

Treatment	Incubation period (hours)					
	0-2	2-4	4-8	8-12	12-24	0-24
100R	32.9	4.9	1.0	13.9	11.0	11.1
16F	29.1	3.1	-7.1	12.1	11.7	9.3
33F	24.4	1.4	-10.3	4.2	9.7	6.0
50F	20.7	4.5	-8.4	-0.9	9.9	5.5
50BT	16.9	9.6	1.8	7.3	12.3	9.9
50BT+PEG	17.4	10.1	2.7	7.1	14.4	11.1
SED	1.22	1.40	2.40	2.75	1.10	0.50
P Flower linear	<0.001	0.541	0.001	<0.001	0.144	<0.001
P Flower quadratic	0.930	0.028	0.013	0.415	0.751	0.095
P BT	0.009	0.004	0.001	0.012	0.047	<0.001
P PEG	0.651	0.721	0.729	0.945	0.078	0.027

SED = standard error of the difference between treatments within time intervals.

P Flower linear = significance of the linear regression for rate of conversion of plant N to NH_3-N against the percentage of clover flower in the herbage.

P Flower quadratic = significance of the quadratic regression for rate of conversion of plant N to NH_3-N against the percentage of clover flower in the herbage.

P BT = significance of the difference between 50F and 50BT.

P PEG = significance of the difference between 50BT and 50BT+PEG.

6.4.6 Volatile fatty acids

The rate of VFA production was rapid for the first 12 hours of all incubations, and was similar for all treatments from 8 to 12 hours (Table 6.5). Increasing percentages of clover flowers (100R, 16F, 33F, 50F) decreased total VFA concentrations at 4 hours (Figure 6.6; $P < 0.001$) due to lower concentrations of all VFA (Figures 6.7 and 6.8), but had little effect on total VFA concentration for the remainder of the experiment.

The main effect of CT in 50BT was evident at 24 h when the total VFA concentration was lower than for all other treatments (3.9 versus 4.1 to 4.5 mM/g DM; Figure 6.6). The CT in birdsfoot trefoil slowed VFA production after 12 h ($P < 0.01$; Table 6.5), and at 24 hours of incubation concentrations were lower for acetate (13%; $P < 0.01$; Figure 6.7), propionate (29%; $P < 0.05$; Figure 6.7), butyrate (12%; $P < 0.05$; Figure 6.8) and minor VFA (14%; $P < 0.05$; Figure 6.8) in 50BT relative to 50BT+PEG. At 12 h, propionate concentrations were greatest in birdsfoot trefoil treatments, with no effects of CT (Figure 6.7).

The molar percentages of acetate (Figure 6.9) ranged from 63 to 72% at 4 hours, dropping to 56 to 59% at 24 hours. Propionate molar percentage (Figure 6.9) ranged from 19 to 30% across treatments and sampling times, butyrate (Figure 6.10) from 7 to 15% and minor VFA (Figure 6.10) from 0.1 to 5.3%. The percentage of VFA as acetate increased linearly ($P < 0.001$) with increasing percentages of flowers at 4 and 8 h. The molar percentage of acetate was lowest in the birdsfoot trefoil treatments from 8 to 24 hours incubation ($P < 0.05$) and was not affected by its CT (Figure 6.9).

Percentages of clover flowers had inconsistent effects on the molar percentage of propionate and butyrate (Figures 6.9 and 6.10). The highest percentage of propionate occurred with 50BT from 8 to 24 hours.

Increasing the percentage of clover flowers caused a significant linear decrease in the percentage of minor VFA at each incubation time (Figure 6.10). 50BT had a higher percentage of minor VFA than 50F at 8 and 24 hours, but birdsfoot trefoil CT did not affect the percentage of minor VFA.

The effects of clover flowers on acetate to propionate ratios (A:P) and acetate plus butyrate to propionate ratios (A+B:P) were inconsistent over time (Figure 6.11) but were higher than 50BT and 50BT+PEG treatments from 8 to 24 hours.

TABLE 6.5 Net rate of VFA production (mM total VFA/g DM/h) from *in vitro* incubations of perennial ryegrass leaf (R) with different percentages of white clover flower (F) or birdsfoot trefoil (BT) with and without PEG.

Treatment	Incubation period (hours)				
	0-4	4-8	8-12	12-24	0-24
100R	0.43	0.10	0.30	0.10	0.19
16F	0.38	0.25	0.21	0.06	0.17
33F	0.34	0.30	0.25	0.05	0.17
50F	0.26	0.35	0.19	0.09	0.18
50BT	0.24	0.36	0.24	0.05	0.16
50BT+PEG	0.26	0.43	0.18	0.09	0.19
SED	0.037	0.066	0.069	0.016	0.007
P Flower linear	<0.001	0.002	0.201	0.375	0.166
P Flower quadratic	0.461	0.325	0.793	0.011	0.098
P BT	0.663	0.877	0.473	0.036	0.102
P PEG	0.724	0.333	0.396	0.018	0.003

SED = standard error of the difference between treatments within time intervals.

P Flower linear = significance of the linear regression for rate of VFA production against the percentage of clover flower in the herbage.

P Flower quadratic = significance of the quadratic regression for rate of VFA production against the percentage of clover flower in the herbage.

P BT = significance of the difference between 50F and 50BT.

P PEG = significance of the difference between 50BT and 50BT+PEG.

FIGURE 6.6 Concentration of total VFA at 4, 8, 12 and 24 hours from *in vitro* incubations of perennial ryegrass leaf (R) with different percentages of white clover flower (F) or birdsfoot trefoil (BT) with and without PEG. Error bars represent SED's.

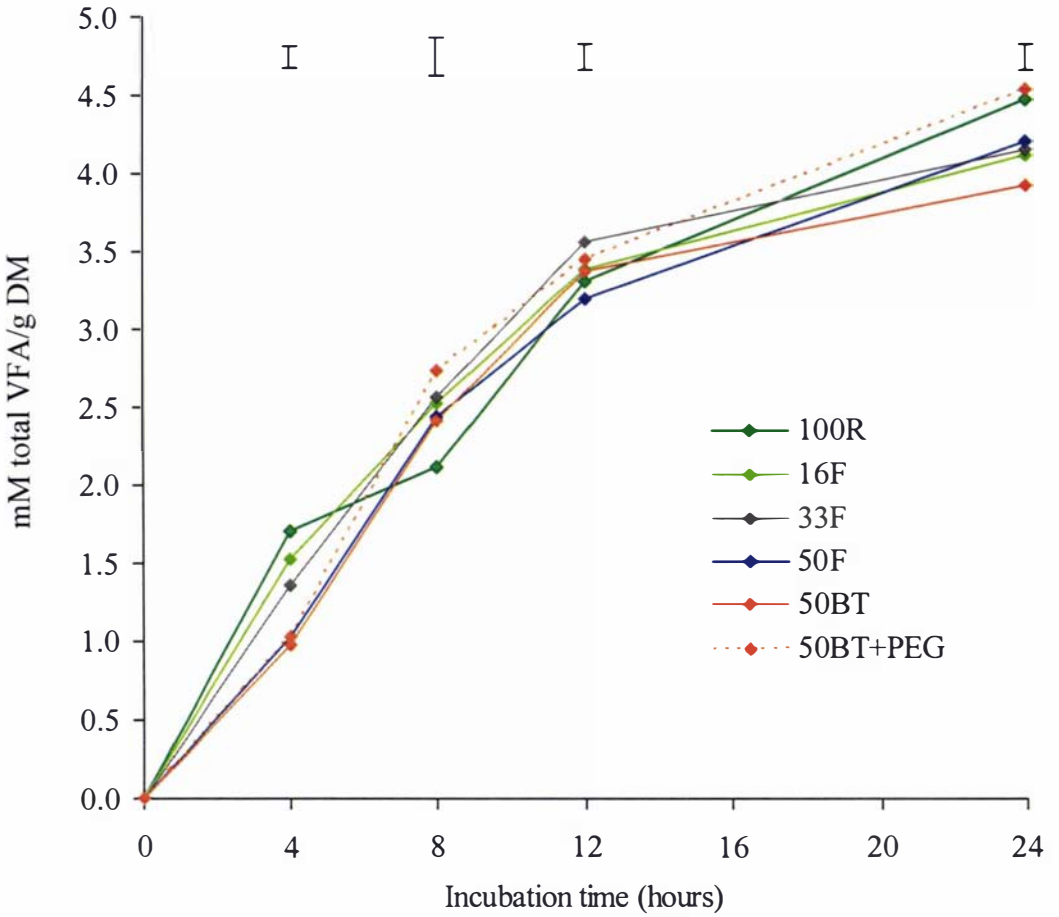


FIGURE 6.7 Concentration of acetate and propionate at 4, 8, 12 and 24 hours from *in vitro* incubations of perennial ryegrass leaf (R) with different percentages of white clover flower (F) or birdsfoot trefoil (BT) with and without PEG. Error bars represent SED's.

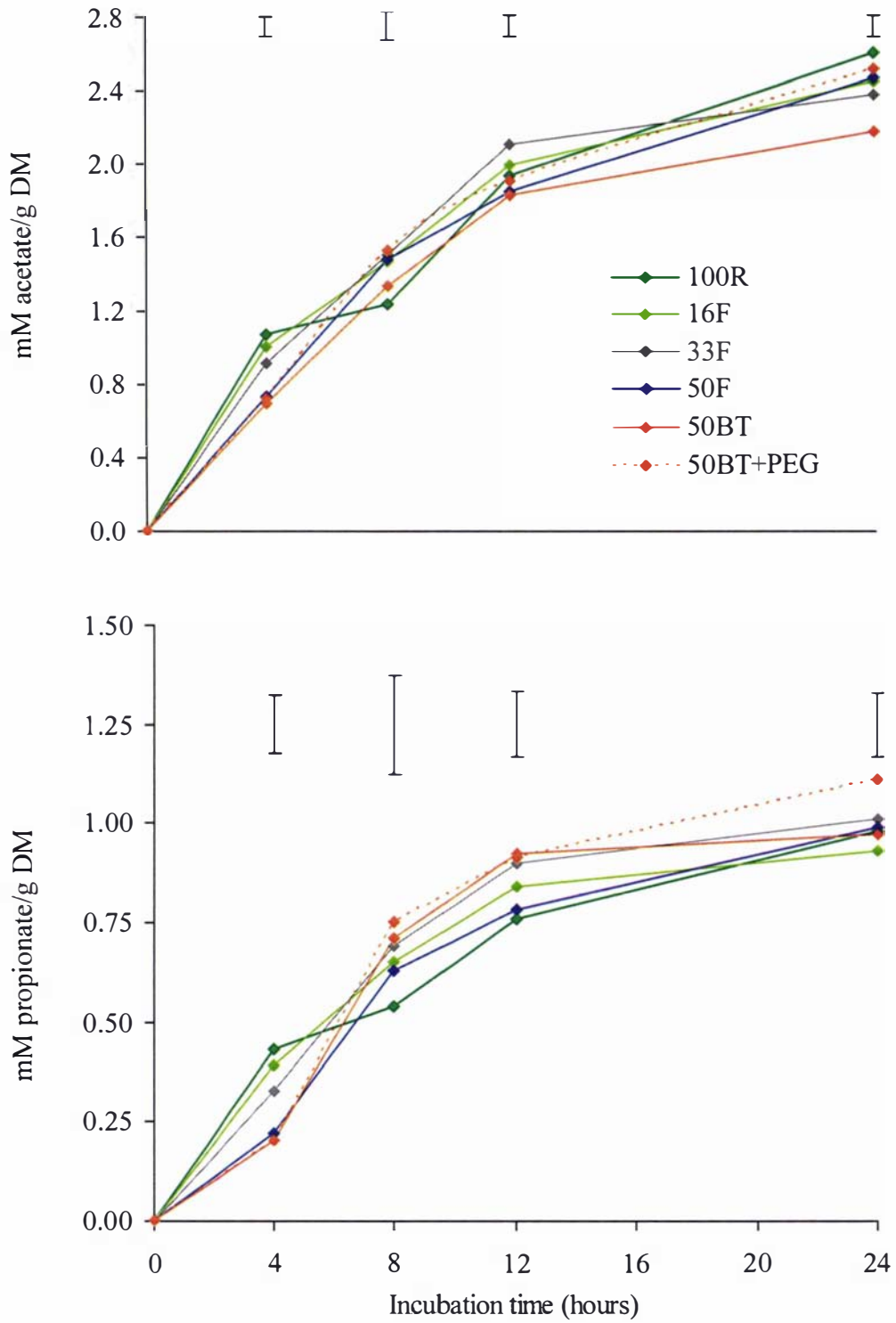


FIGURE 6.8 Concentration of butyrate and minor VFA (isobutyrate, valerate, isovalerate) at 4, 8, 12 and 24 hours from *in vitro* incubations of perennial ryegrass leaf (R) with different percentages of white clover flower (F) or birdsfoot trefoil (BT) with and without PEG. Error bars represent SED's.

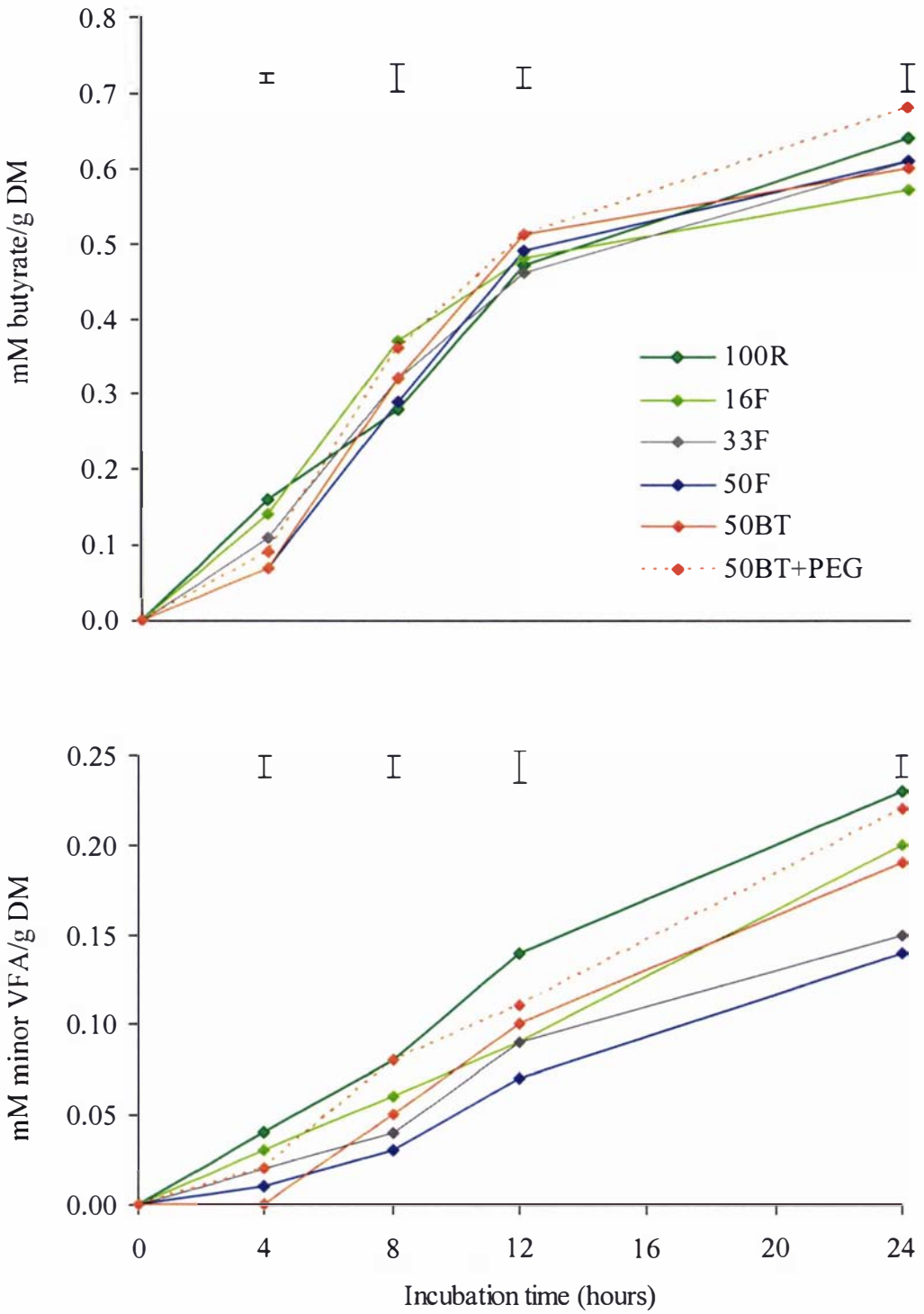


FIGURE 6.9 Molar percentage of acetate and propionate at 4, 8, 12 and 24 hours from *in vitro* incubations of perennial ryegrass leaf (R) with different percentages of white clover flower (F) or birdsfoot trefoil (BT) with and without PEG. Error bars represent SED's.

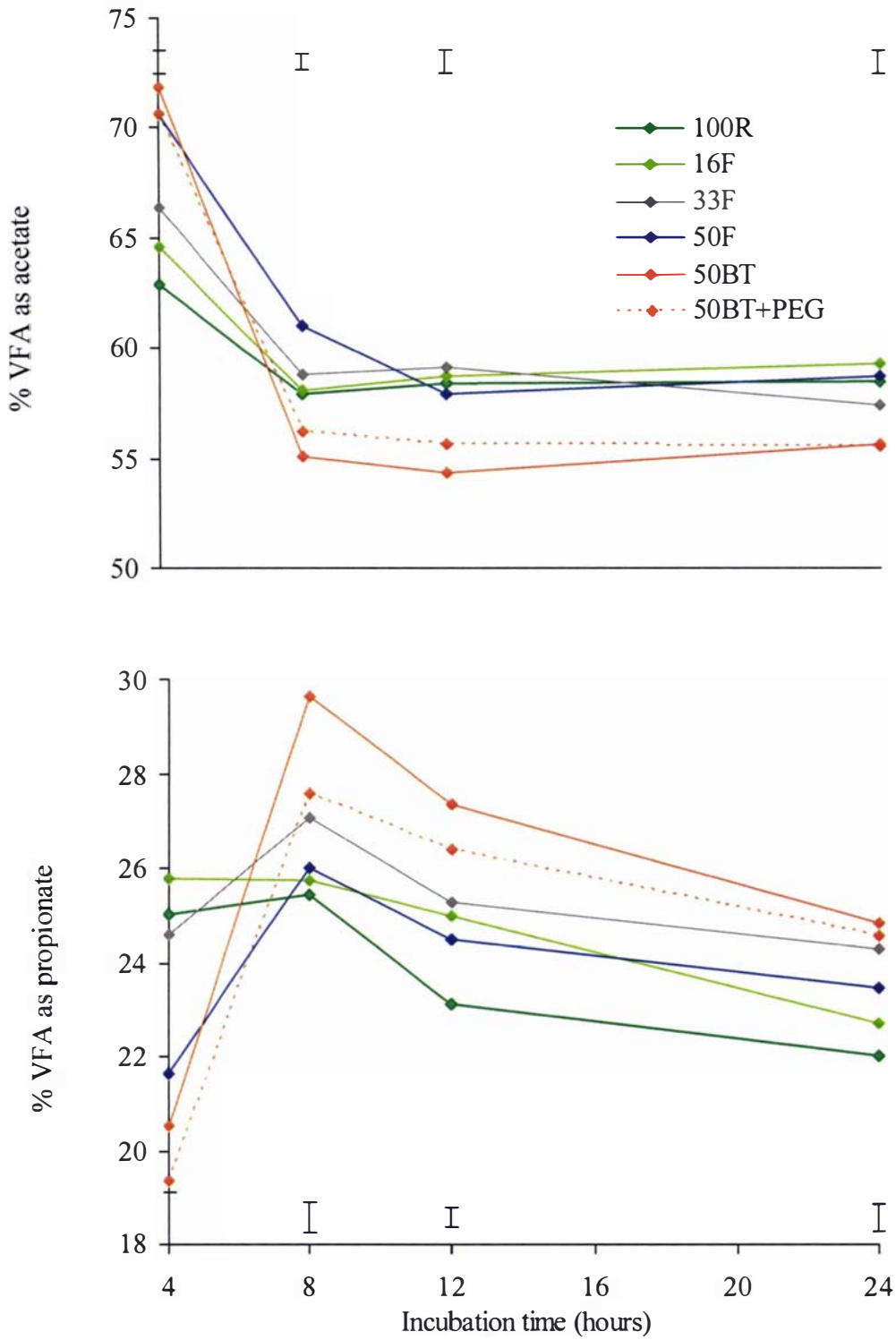


FIGURE 6.10 Molar percentage of butyrate and minor VFA (isobutyrate, valerate, isovalerate) at 4, 8, 12 and 24 hours from *in vitro* incubations of perennial ryegrass leaf (R) with different percentages of white clover flower (F) or birdsfoot trefoil (BT) with and without PEG. Error bars represent SED's.

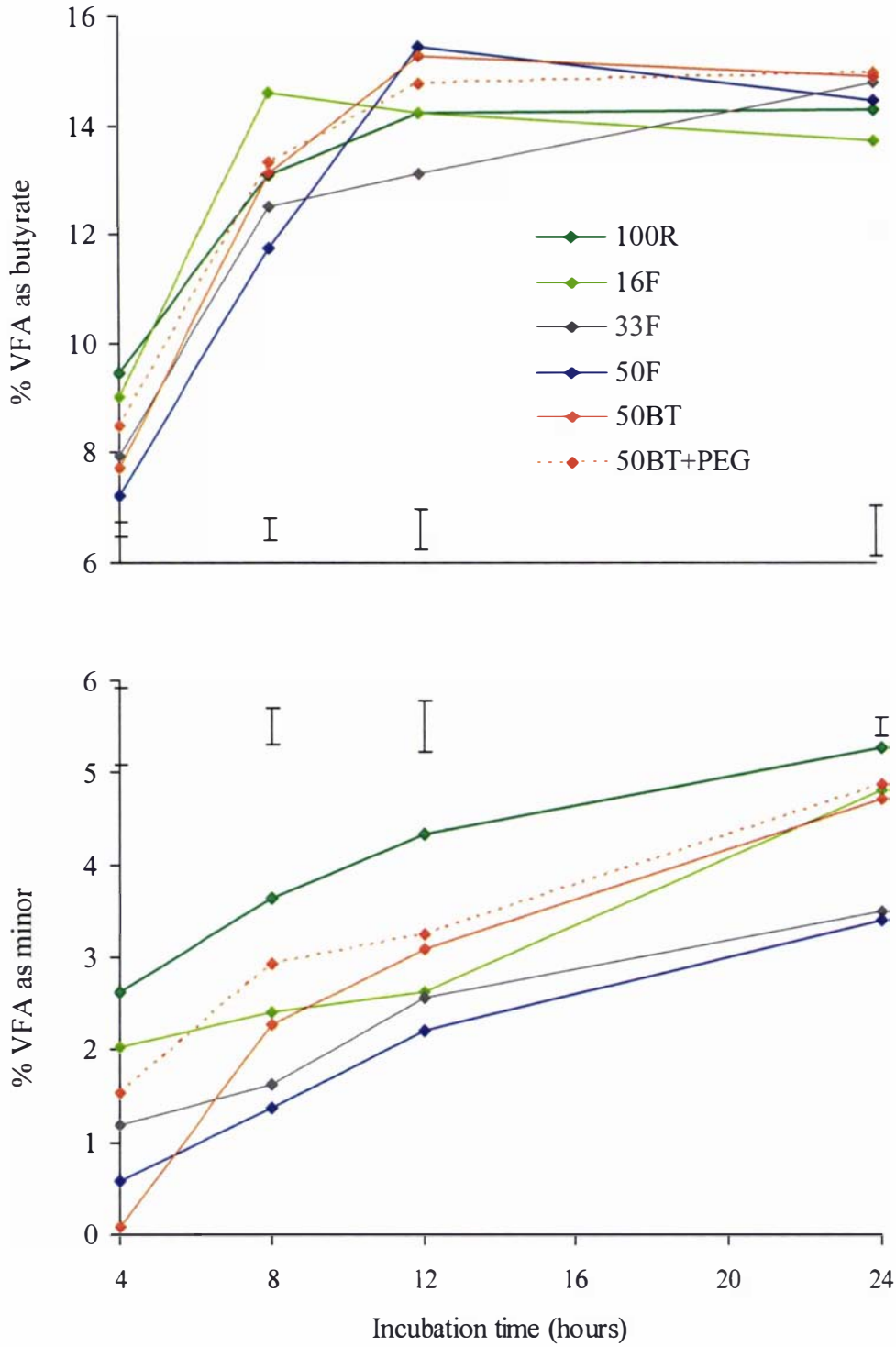
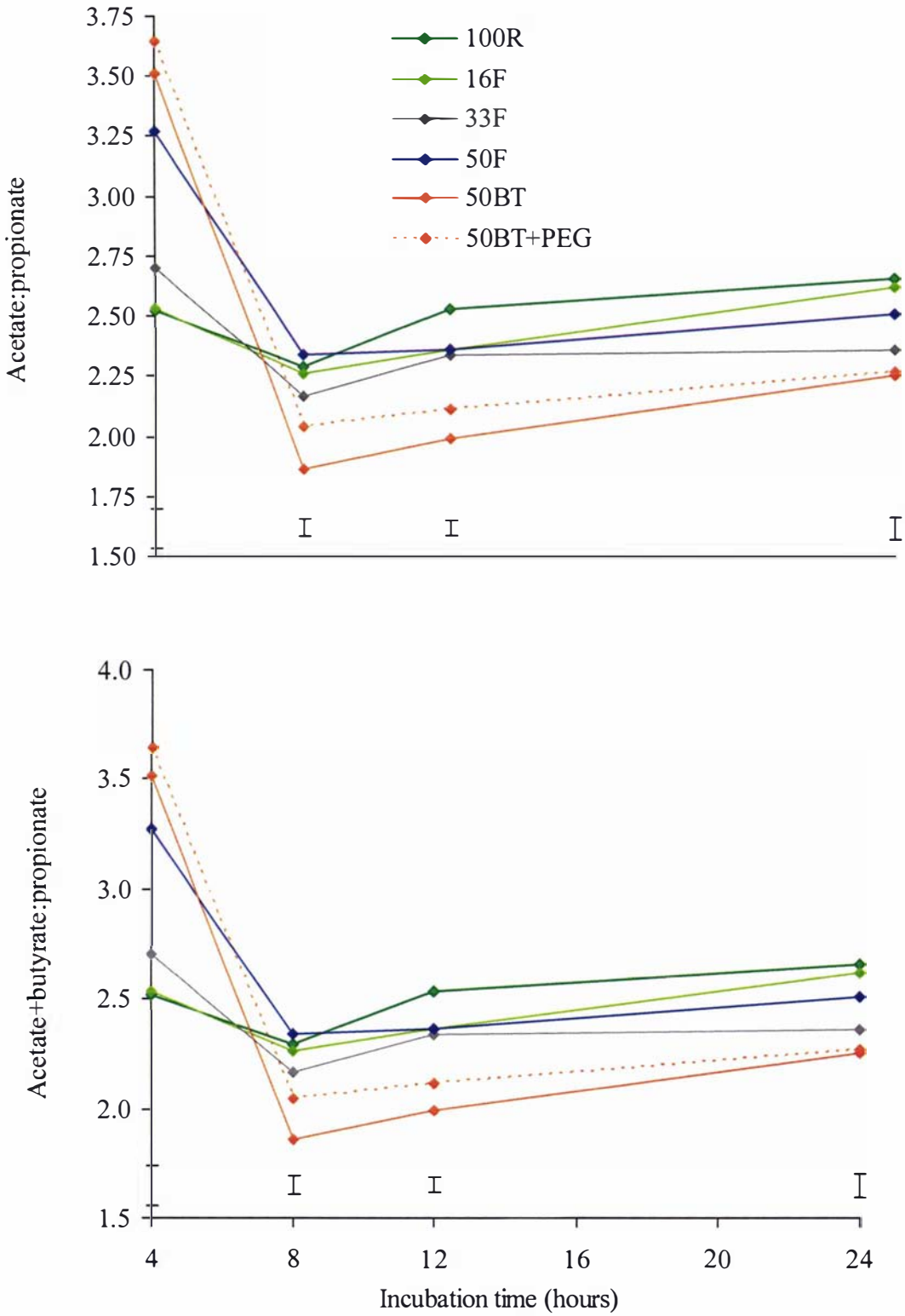


FIGURE 6.11 Ratio of acetate to propionate and acetate+butyrate:propionate at 4, 8, 12 and 24 hours from *in vitro* incubation of perennial ryegrass leaf (R) with different percentages of white clover flower (F) or birdsfoot trefoil (BT) with or without PEG. Error bars represent SED's.



6.5 DISCUSSION

This experiment has shown differences between birdsfoot trefoil herbage and white clover flowers in their products of digestion when mixed with perennial ryegrass leaf. Ryegrass mixed with clover flowers resulted in lower soluble protein and ammonia concentrations than when mixed with birdsfoot trefoil, partly due to the lower crude protein concentration in the white clover flowers. The percentage of plant N appearing as ammonia N decreased linearly with increasing percentages of clover flower and values for all flower treatments were lower than 50BT from 8 to 24 h of incubation. The CT in 50BT reduced soluble protein by 20% at 0 h, but had little effect on ammonia concentrations throughout the experiment. All treatments had similar total VFA concentrations, but the A:P ratios were lowest in birdsfoot trefoil treatments, partly due to its CT.

6.5.1 Feed composition

Crude protein concentrations for all treatments exceeded the 180 g/kg DM recommended for cows producing 20 kg milk/day, and only the birdsfoot trefoil treatments contained more than the 240 g/kg DM recommended for cows producing 30 kg milk/day from pasture (Kolver 2000). 50F was the only treatment with CT concentrations within the range of 20 to 40 g/kg DM considered optimum for ruminant nutrition (Aerts et al. 1999). Concentrations in pure birdsfoot trefoil were also within this range, and similar to previous reports (Waghorn et al. 1998). The birdsfoot trefoil used in this experiment was short leafy regrowth and even when mixed with ryegrass leaf, NDF was below optimum (35% of DM) for pasture diets (Kolver 2000).

The particle size distributions of material used for *in vitro* incubations (Table 6.2) were similar to the rumen contents of cows fed perennial ryegrass or lucerne (*Medicago sativa*) (Table 5.8), although there were smaller proportions of DM < 0.25 mm for treatments without BT. The smaller mean particle size and greater release of DM into solution from minced birdsfoot trefoil than from minced clover flowers or ryegrass leaves is probably due to the low fibre content of the birdsfoot trefoil or the passage of intact white clover florets through the mincer. Large particles require further breakdown by chewing to release cell contents and this will increase the surface area available for attachment of fibre-degrading bacteria (Weimer 1996). Few feed particles larger than 2 mm pass out of the rumen of cows (Waghorn 1986) and this can limit feed

intake and animal production. Dado & Allen (1994) showed that more time is spent chewing feed with a high NDF content. The minced plant material used for incubation in this study was similar to chewed forages, so relative rates of digestion should match *in vivo* digestion.

6.5.2 Soluble protein and ammonia

The release of plant protein into solution following chewing increases its susceptibility to enzymatic degradation in the rumen (Mangan 1972; Nugent et al. 1983; Wallace 1983), forming peptides, amino acids, ammonia, and the minor VFAs. Although some ammonia is incorporated into microbial protein, excretion of excess ammonia as urea has an energy cost to the animal (Martin & Blaxter 1965; Twigge & van Gils 1984) and can be detrimental to the environment.

The low percentage of soluble protein at 0 h for all treatments (<7.5% of plant protein) was similar to the results reported in Chapter 5. The extent of net conversion of plant protein to ammonia appears to be a function of plant CP concentration, with the values for 100R higher than that from ryegrass containing only 15% CP in the DM (Burke 2004). Conversion of plant N to NH_3-N in 50BT was similar to that reported for birdsfoot trefoil (Burke 2004).

The decrease in soluble protein concentration and lower conversion of plant-N to NH_3-N with increasing percentages of clover flowers relative to ryegrass leaf (Figures 6.4, 6.5) is similar to that reported in Chapter 5 with white clover flowers and clover leaves. This relationship is a function of the lower protein and higher fibre concentrations in clover flowers relative to either ryegrass or clover leaves, with little effect due to the CT in flowers.

The higher ammonia concentration and percentage of plant N appearing as ammonia-N for 50BT compared to 50F is probably a consequence of its higher crude protein concentration (Table 6.1) and a greater release of soluble DM (including protein) from minced birdsfoot trefoil compared to clover flowers (Table 6.2). The net release of ammonia from all treatments exceeded utilisation for the duration of the experiment.

When the effects of birdsfoot trefoil CT were removed with PEG there was 20% more crude protein solubilised at 0 h. In contrast, results from Chapter 5 (summarised in Table 6.6) show white clover CT (from a mixture of 50% flowers and 50% leaves) had no effect on protein concentrations at 0 h suggesting either a less reactive CT or less cell and vacuole rupture and CT release. However, at 2 h, clover flower CT reduced soluble protein concentration by 20%, whilst there were no effects of CT from BT, suggesting a slower release of CT from white clover flowers than from birdsfoot trefoil. Both types of CT had similar effects on soluble protein for the remainder of the incubations.

Some evidence suggests high molecular weight CT interacts more strongly with protein than CT of low mean molecular weight (Beart et al. 1985; Horigome et al. 1988; Kawamoto et al. 1996). The higher molecular weight of white clover CT (approximately 3100 daltons; Sivakumaran et al. 2004) compared to that from BT (1900 daltons; Foo et al. 1996), combined with its higher CT concentration (52 versus 31 g/kg DM) indicate white clover CT should have precipitated more protein than that from BT. The effect of CT on proteolysis may be more responsive to differences in their chemical composition than their molecular weight. Aerts et al. (1999) attributed the greater inhibition of proteolysis by CT from lotus major (*Lotus pedunculatus* Cav.) compared to birdsfoot trefoil to the higher proportion of prodelphinidin sub-units. White clover floral CT is composed solely of prodelphinidin sub-units (Foo et al. 2000), and inhibition of proteolysis should be greater than from birdsfoot trefoil on a molar basis. Any conclusion concerning relative reactivity of the two CT sources evaluated here and in Chapter 5 is complicated by differences in protein and CT concentrations as well as differences in cell and vacuole rupture for the two forages.

The CT in birdsfoot trefoil did not affect ammonia concentrations during the first 12 hours of incubation with 50% ryegrass (Table 6.6), suggesting either insufficient activity of the CT, or that changes in proteolysis were balanced by changes in microbial demand for ammonia. The absence of effects on proteolysis were supported by similar concentrations of minor VFA (derived from amino acid degradation) for BT treatments with and without PEG. Reduced ammonia and minor VFA concentrations attributed to white clover CT and birdsfoot trefoil CT at 12 and/or 24 hours (Table 6.6) demonstrate some CT activity, but *in vivo* effects would be minimal as rumen residence time is likely to be 12 hours or less.

TABLE 6.6 Change (%) in soluble protein, ammonia and minor VFA (isobutyrate, valerate, isovalerate) concentrations *in vitro* attributed to CT in a 50% mixture of white clover flower and leaf, and a 50% mixture of birdsfoot trefoil with perennial ryegrass leaf. Data are calculated from the difference between treatments with and without polyethylene glycol.

Time	Birdsfoot trefoil CT	White clover CT[1]
Soluble protein		
0	-20%*	+9% NS
2	-0.7% NS	-20%**
4	-13%**	-14%**
8	-7% NS	-1.3% NS
12	+8% NS	-2.4% NS
24	+0.8% NS	-0.6% NS
Ammonia		
2 h	-6.5% NS	+24%**
4 h	-5.6% NS	+14% NS
8 h	-8.3% NS	-6.5% NS
12 h	-4.7% NS	-45%**
24 h	-8.7%*	-4.3% NS
Minor VFA		
2 h	No data	+8.7% NS
4 h	-100% NS	+66% NS
8 h	-34% NS	-1.2% NS
12 h	-7.1% NS	-21 %*
24 h	-16%***	-19%***
CT concentration of mixture:	15.7 g/kg DM	26.2 g/kg DM
Crude protein concentration of mixture:	269 g/kg DM	204 g/kg DM

[1] Data sourced from Chapter 5, NS = CT effect not significant, * = CT effect significant at $P < 0.05$, ** = CT effect significant at $P < 0.01$, *** = CT effect significant at $P < 0.001$.

The transfer of CT to react with protein in other plants in the diet requires the concentrations of free CT to exceed the binding capacity in the CT-containing plant (Barry & Manley 1986), and the protein from the CT-free plant to be available in the rumen at the same time as the CT. When birdsfoot trefoil was mixed with pasture (1:2 ratio, DM basis) and fed to sheep (22.1 g CT/kg dietary DM) the CT did not affect rumen ammonia concentration (Waghorn & Shelton 1997). These authors homogenised a pasture/birdsfoot trefoil mixture in liquid nitrogen for *in vitro* incubation and demonstrated reduced ammonia concentrations due to the CT. The finely homogenised preparation demonstrated the importance of cell rupture to elicit a CT effect, but it was not representative of chewed forage or the particle size preparation used here.

6.5.3 Volatile fatty acids and pH

All incubations maintained a pH above 5.7 and were representative of normal rumen function. The rapid drop in pH in 100R was due to a rapid initial fermentation and VFA production in this treatment (Table 6.5), and the small change in all treatments between 12 and 24 hours of incubation was associated with a reduced rate of VFA production. Addition of white clover flowers resulted in a more consistent VFA production over the first 12 hours of incubation, and the percentage of acetate at 4 h increased with increasing flower contents. The effects of flowers on A:P and A+B:P ratios at other times were inconsistent.

Comparisons between *in vitro* and *in vivo* data should be undertaken with care, because concentrations *in vivo* are a balance between production, absorption and rumen digesta volume. Nevertheless, CT from lotus major (Barry et al. 1986) and sainfoin (McMahon et al. 1997) have lowered rumen VFA concentrations, but when the CT from lotus major were diluted to 18 g CT/kg DM (similar to the CT concentration of 50BT) by feeding with ryegrass, VFA concentrations were not affected (Waghorn & Shelton 1995). The minor effects of CT in BT incubated with ryegrass on VFA production and molar percentages of the major VFA complement *in vivo* findings with sheep fed BT as a sole diet (Wang et al. 1996a) or with ryegrass (Waghorn & Shelton 1997).

VFA form the primary energy source for ruminants, accounting for up to 85% of energy absorbed from the digestive tract (Agriculture and Food Research Council 1998). The efficiency of metabolisable energy use for animal production is increased with

decreasing A:P ratios (Waghorn & Barry 1987). The lower A:P ratios arising from incubations containing birdsfoot trefoil and the inconsistent effects of increasing clover flowers suggests a higher nutritive value of mixed birdsfoot trefoil and perennial ryegrass diets compared to ryegrass alone or ryegrass with high flowering white clover. The high nutritive value of birdsfoot trefoil has been demonstrated when fed as a sole diet to lactating cows (Harris et al. 1998; Woodward et al. 1999) and sheep (Wang et al. 1994, 1996a,b; Douglas et al. 1999). There has been no significant production advantage to lactating cattle fed white clover with high compared to low proportions of flowers (Chapter 4; Stockdale & Dellow 1995; Stockdale 1997).

6.6 CONCLUSIONS

Increasing percentages of clover flowers relative to ryegrass leaves reduced protein and ammonia concentrations, and reduced the percentage of plant protein converted to ammonia, with inconsistent effects on VFA production. Birdsfoot trefoil was not as effective at reducing proteolysis as white clover flowers, but it lowered acetate to propionate ratios, suggesting a higher nutritive value in ryegrass-based diets than white clover flowers.

CHAPTER 7

General discussion and conclusions

CHAPTER 7: General discussion and conclusions

7.1 INTRODUCTION

Increasing the proportion of white clover (*Trifolium repens* L.) in temperate pastures normally increases dairy cow milk production (Harris et al. 1997). However, the response is limited in part because of the incidence of bloat and also the requirements for elimination of excess dietary crude protein. The ability of condensed tannins (CT) to prevent bloat and improve protein utilisation can lead to substantial improvement in the performance of animals fed legumes containing moderate concentrations of these compounds (Waghorn et al. 1998). These effects are mediated through the ability of CT to bind to plant proteins and protect them from degradation to ammonia in the rumen. Animal responses vary with the type of CT and the dietary concentration of CT and other nutrients. Plants with CT may also have poorer agronomic performance than pasture plants without CT (Waghorn et al. 1998).

The aim of this thesis was to evaluate the performance of a white clover selected for increased floral CT concentrations, compared to Grasslands Huia white clover as a control. White clover forage has very low CT concentrations, as CT is predominantly produced in the flowers. High tannin (HT) white clover was selected for increased floral CT concentrations, a longer flowering period and a higher density of flowers. Agronomic performance was assessed over 2 years, with particular emphasis on seasonal herbage accumulation, floral CT concentrations, flower and growing point densities and plant morphology. Animal performance was evaluated in five grazing experiments to determine the effects of HT clover on rumen digestion, milk production and bloat. Small differences in the concentration of rumen metabolites between treatments and the lack of a milk production response prompted closer examination of the effects of white clover flowers on feed digestion in the rumen in two *in vitro* incubation experiments.

The results of individual experiments are discussed in detail in chapters 3 to 6. This chapter considers some of the limitations of the techniques used in these experiments,

then brings together the major findings of the four experimental chapters and their implications for plant breeding and the use of high tannin white clover on New Zealand dairy farms. Some future research required to answer questions arising from this work is also discussed. General conclusions are then drawn on the value of white clover flowers as a source of condensed tannins.

7.2 EVALUATION OF THE METHODOLOGY USED IN THE CURRENT RESEARCH

7.2.1 Control cultivar

When assessing new forage cultivars, the selection of a control treatment should be the most promising alternative cultivar for the particular situation (Reed 1994). The control in this experiment, Grasslands Huia, has been superseded in agronomic performance by more recent cultivars (Woodfield et al. 2001), and is no longer commonly sown in New Zealand dairy pastures. If HT had performed better than Huia it could not be concluded that it is necessarily the best option for dairying. However, Huia has been widely used as a control, so there is much information available comparing its performance with other cultivars; this situation also enhances the reliability of comparisons of different experiments with the same control (Thomas & Laidlaw 1981).

The breeding of HT began with selection for increased floral CT concentration within Huia lines (Section 2.4.4). The use of Huia as a control was further justified by its similar genotype to HT except in the traits being tested of flowering and floral CT concentration. This reduced any confounding effects that other cultivars may have had on agronomic performance.

7.2.2 Pure sward assessment

This research assessed the performance of HT white clover in pure clover swards. Pure swards of white clover are not often used for dairying in New Zealand because of the likelihood of bloat, low herbage production and weed control issues. Evaluation of HT white clover in pure swards therefore does not represent normal farm practice. Snaydon (1978) also noted that differences in animal performance obtained in pure swards may

not occur in mixed swards due to dilution of the active components, and interactions with other sward components.

New Zealand dairy farmers have recently experienced difficulties in maintaining clover contents in mixed pastures high enough for optimum animal performance (Ettema & Ledgard 1992; Bateup et al. 2001). Having white clover with 20-40 g CT/kg DM may enable white clover to be safely grazed (without the risk of bloat) separately from grasses, minimising the metabolic cost of ammonia detoxification, while providing enough clover in the diet to sustain high animal production.

Pure sward assessments were therefore used to avoid complications due to varying percentages of different species in the two clover treatments (Minson et al. 1960). Mixed sward experiments were to be undertaken if HT showed benefits in pure swards. However, this research has shown significant differences in the growth, morphology and persistence of the two clovers that would have been difficult to monitor in mixed swards. HT clover swards generally had higher weed contents and were less productive in monoculture than Huia, and did not provide extra benefits to animal production. Thus, mixed sward studies were not undertaken.

7.2.3 Measurements of plant performance and CT concentration

One of the major objectives of this research was to compare the agronomic performance of HT white clover to a standard white clover cultivar (Grasslands Huia), as plants containing high concentrations of secondary metabolites (such as CT) typically have lower relative growth rates than those with low concentrations (Lambers & Poorter 1992).

When designing this experiment, a balance was required between the number of treatment replicates and the amount of resources needed to undertake measurements within these replicates, with the goal of reducing the coefficient of variation about the treatment mean as much as possible (Thomas & Laidlaw 1993). For measurements of plant performance, the experiment was designed as a randomised block with three 1 ha replicates per treatment. Increasing the number of replicates to four would have increased the precision and ability to detect significant differences between the two clover treatments. However, there was a fixed land area available for the research. As

the same land area was also required for the measurement of animal performance, replicates had to be fenced and managed separately. Smaller treatment areas would have made grazing and management more difficult.

Herbage dry matter accumulation was assessed by quadrat cuts in grazing exclosure cages, relocated after each cut. Cages were located in areas free from weeds, so that the resulting herbage accumulation represented that achievable without competition from other species. This methodology would have overestimated the actual clover dry matter grown in the whole paddock. Annual clover herbage accumulation was lower for HT than Huia, and because of the higher weed content of HT, the differences between treatments would have been greater than those measured. Similarly, clover morphology turves were collected from areas free of weeds, which again may have been biased in favour of HT performing better than if areas containing weeds were included.

Another consideration is whether or not the standard used for CT analysis was accurately predicting the CT concentration in white clover flowers. While use of commercially available standards allows comparison of results amongst studies, the chemical composition of these standards is likely to differ appreciably from the test species, resulting in bias of measurements. Because tannins are made up of a diverse group of polyphenols, standards should be purified from the species of interest (Kraus et al. 2003). This research used lotus major (*Lotus pedunculatus* Cav.) as a standard for CT quantification. White clover CT was not used as a standard because of its low concentrations in forage and therefore expense and difficulty to obtain sufficient quantities for purification. However, the lotus standard enabled relative values to be compared for the two clovers and showed large variation between sampling dates but no effect of selection on CT concentration.

7.2.4 Measurements of animal performance

Animal performance was evaluated in a series of short-term grazing experiments. Statistically significant ($P < 0.05$) differences in milk production or composition were not detected in these experiments. Milk yield can be accurately measured, and the main source of error in milk production is the variability between animals. This is attributed to differences between animals in feed intake and its nutritive value, and the efficiency of use of these nutrients for milk production. Animal numbers per treatment were

calculated from the standard deviation of milk yield from cows grazing white clover in other experiments, and the desired minimum detectable difference at the 5% level of probability. Recalculation using the standard deviation in milk yield from the first experiment would have provided a check on the validity of these numbers and allowed for adjustment of numbers in subsequent experiments if required.

Accurate measurement of pasture dry matter intake by grazing animals is extremely difficult. The alkane marker technique was intended to provide an estimate of intake for individual cows, as opposed to pre and post-grazing herbage mass estimation, which only determines daily herd averages. The alkane technique has been validated indoors for pasture-fed cows (Dove & Mayes 1991), but proved to be erroneous in this and other experiments (Chapter 4).

Determining the effects of HT white clover on bloat proved difficult, as the incidence and severity of bloat was low in both treatments. The occurrence of bloat on farms is unpredictable, but tends to be most common when foliage is lush in spring. Clover swards always had low dry matter (DM) percentages during the experiments (< 17%) and bloat measurement periods were included in each experiment to increase the chances of detection. Flowers were also present at each bloat measurement period, decreasing the likelihood of bloat because they contained CT. Increasing the number of days that bloat observations were made would have increased the likelihood of bloat detection, as a minimum period of 24 to 48 hours feeding bloat-inducing feed is normally required before it occurs (Reid et al. 1975). Bloat is also more likely to occur when cows are given a fresh pasture break after a period of limited feed supply (Waghorn 1991). For this experiment cows were always fully fed to ensure differences in animal performance were mainly due to nutritional quality and were not confounded by differences in the quantity of herbage available.

7.2.5 *In vitro* techniques for determining nutritive value

The use of *in vitro* techniques for measuring the products of rumen digestion enables a rapid and cost effective evaluation of forages. *In vitro* incubations enable good control of incubation conditions and remove between animal variation, but *in vitro* results are not always repeatable *in vivo* (Waghorn & Shelton 1997). The *in vitro* technique used in this research was a batch culture, with forage added at the start of the incubations,

and the products of digestion were allowed to accumulate in the system. The constraints of batch culture were acceptable for the evaluations carried out here, but dual flow continuous culture systems (Hannah et al. 1986) more accurately simulate solid and liquid flow out of the rumen.

The cause of less plant crude protein appearing as ammonia for white clover flowers than leaves was not quantified in this research. *In vitro* measurements of plant dry matter and protein disappearance may have enabled better interpretation of these treatment differences. However, larger particles and a lower percentage of DM in solution following mincing (Table 5.2) indicated less cell rupture (and therefore release of cell contents) for flowers than leaves. The passage of flower petals through the mincer undamaged was also noted. This indicates digestion of clover flowers may be slow or incomplete in cows, reducing the release and therefore impact of CT.

7.3 BREEDING WHITE CLOVER FOR INCREASED FLORAL CONDENSED TANNIN

HT white clover was bred with the aim of producing condensed tannin concentrations high enough to improve dairy cow performance relative to cows fed standard white clover cultivars. Because white clover produces CT primarily in its flowers, the breeding programme involved first selecting white clover with increased floral CT concentrations and then incorporating genetics from white clover with prolific flowering to increase the duration and intensity of flowering. Initial testing of HT white clover showed a 26% increase in floral CT concentrations over the Huia line it was selected from (Figure 2.5)

Regular monitoring of floral CT concentrations in the field revealed no difference between treatments, but increased flowering in HT led to higher forage CT concentrations for much of the experiment. The agronomic measurements (Chapter 3) indicated that increased flowering was detrimental to the persistence of HT clover. Flower heads were also of lower nutritive value (lower digestibility, crude protein and ME content) than the rest of the harvestable portion of the clover plant (Chapters 4) and may lead to a less beneficial supply of volatile fatty acids for animal production than white clover leaves (Chapters 5).

Future breeding to increase CT concentrations in white clover should concentrate on increasing the concentration of CT in the flowers, rather than increasing flowering. Increased number of flower heads per plant (Brigham & Wilsie 1955) has been used to increase white clover seed yields, but this is the first white clover selected for increased floral CT concentration. Miller & Ehlke (1997) indicated that CT concentration in birdsfoot trefoil is controlled by additive gene effects, and that mass selection would be an effective way to increase or decrease herbage CT concentration. Vaillancourt et al. (1986) studied the inheritance of seedcoat CT concentration in four lentil (*Lens culinaris* Medik.) lines and obtained high heritability estimates (66.4 ± 4.4%). Earlier generations in the breeding programme of HT white clover (Figure 2.5) revealed some large differences between clover lines in floral CT concentration, but this failed to be maintained through the seed multiplication stage. Reasons for the loss of floral CT and the control of floral CT concentrations are not known.

Potential problems with breeding pasture plants for improved nutritive value include cultivar x maturity interactions and cultivar x environment interactions (Clements 1970). In HT white clover, selections for high CT concentrations were only made twice per year, and at one stage of flower maturity. These selection gains may not have been fully expressed at other stages of flower development, for swards versus spaced plants, for mixed versus pure swards, and in different environments (Hutchinson & Clements 1987). Condensed tannins in other plant species vary in response to soil fertility, climate, stage of plant development and competition with other species (Barry & Manley 1986; Lees et al. 1995; Carter et al. 1999; Wen et al. 2003) but control of CT expression is not well defined.

This research showed large seasonal variation in floral CT concentrations and the proportion of flower heads in the sward. Peak floral CT concentrations appeared to be related to the stage of flower maturity, increasing to full bloom, and decreasing with senescence. In the breeding programme, only flowers in full bloom were selected for CT analysis and any senescing florets were removed. However, under grazing a wide range of flower developmental stages were present at any one time. Climate and grazing interval affect the speed of flower development and the amount of time available to mature and therefore are able to influence sward CT concentrations.

These experiments have illustrated the risks in extrapolating results from one evaluation procedure to other situations. Because CT monitoring in this experiment ran over 2 years there was an opportunity to cover a range of environmental conditions. Floral CT concentrations in HT failed to show a consistent increase over that in Huia white clover, and so would be unlikely to do so under grazing in other locations. Given the vast variation between sampling dates, and similarity between clover lines within sampling dates, it appears that further genetic selection for increased floral CT within white clover would be difficult without an understanding of the causes of this variation.

7.4 WHITE CLOVER FLOWERS AS A SOURCE OF CONDENSED TANNINS

7.4.1 Condensed tannin concentration and plant performance

For HT white clover to benefit animal performance, appropriate CT concentrations within the plant must be maintained, and the plant must make up a sufficient proportion of the feed on offer to ensure optimum dietary CT concentrations.

Although the protein–binding capacity of CT differs between plant species, the optimum dietary concentration of CT in forage legumes may be about 20 to 40 g/kg DM (Aerts et al. 1999), although more astringent CT could achieve similar benefits at lower concentrations (Mangan 1988). Clover CT concentrations were higher in HT than Huia, but maximum concentrations were only 12 g/kg DM. There was also large variation in concentrations within and between years due to the seasonality of flowering and large fluctuations in CT concentrations within flower heads (Table 3.6).

Higher clover CT concentrations in HT than in Huia were achieved predominantly through increased flowering. Increased flowering reduces the number of sites available for stolon production. In temperate climates such as New Zealand, white clover persists primarily through vegetative production of new stolon branches (Williams 1987b). The persistence of HT clover was inferior to that of Huia. Lower stolon growing point densities in HT led to gaps in the sward, enabling the invasion of weeds and competition from other white clover genotypes.

HT white clover showed less potential as a forage cultivar for grazing than Huia white clover because of lower spring and summer DM production. HT swards also had more weeds, so without regular herbicide application, HT white clover would soon become a small proportion of the diet. When grown with grasses in mixed swards, the contribution of HT to the diet would be further diluted.

The contamination of HT swards with other white clover genotypes contributed to the loss in treatment differences in sward CT concentrations within 2 years of sowing. The benefits of plants having improved nutritive value cannot be realised unless they can be successfully established and maintained long enough to outweigh the costs of pasture renewal. This is unlikely to occur unless new genotypes are also well adapted to the environment and can out-compete any white clover germinating from the large source of viable buried seed in dairy pastures (Suckling & Charlton1978; Ledgard et al. 1998).

7.4.2 Animal performance

Condensed tannins improve animal performance by binding with plant proteins in the rumen. The degree of protection from bloat and rumen proteolysis afforded by CT is dependent on its concentration and structure, with higher concentrations of CT able to bind and protect more protein in the rumen. Benefits of CT depend on protein digestion in the intestine, with excessive or astringent CT reducing amino acid absorption (Waghorn et al. 1994a).

Dietary CT concentrations of cows fed HT white clover only diets were from 7 to 12 g/kg DM in 4 out of the 5 experiments, but only 0.6 g/kg DM in the final experiment. Comparable concentrations for Huia white clover diets were 2 to 5 g/kg DM, and 0.6 in the final experiment. The differences in dietary CT concentration between treatments in the first 4 experiments elicited a small difference in rumen ammonia concentrations (Table 4.8), although these changes did not affect milk production or composition (Table 4.11). This finding was mainly attributed to low CT concentrations, but the higher proportion of flowers in the HT diet may also have been less beneficial to animal production (Chapter 5).

White clover is normally fed in mixed pastures with perennial ryegrass (*Lolium perenne* L.) in New Zealand, and the clover content of most dairy pastures averages less than

20% of DM (Ettema & Ledgard 1992). The poor agronomic performance of HT white clover would result in low clover contents in dairy pastures and cows would derive less benefit than from a more productive and persistent cultivar, irrespective of CT concentrations.

The CT concentrations required for bloat prevention have may be much lower than requirements for optimal animal production. Li et al. (1996) proposed 5 g CT/kg DM for bloat safety when feeding legumes and Stockdale (1994) reported the incidence of bloat in dairy cows to be reduced when flowers were 5% or more of the DM of a white clover diet. These guidelines were often met or exceeded with HT white clover, and bloat incidence was low, but not eliminated. Bloat was not measured in early spring, the highest risk period in New Zealand. However, flower densities, and hence sward CT concentrations were low at this time compared to summer to mid-autumn when bloat was detected. HT white clover is therefore unlikely to protect cattle from bloat during the main risk period.

7.4.3 Effect of white clover flowers on rumen digestion

Increased CT concentrations in HT swards were primarily the result of a higher ratio of flowers to leaves (Chapter 3). Flowers differ in nutritive value from leaves, not only because of the presence of CT, but also because of lower protein concentrations, digestibility and metabolisable energy content. All of these factors affect the rate of fermentation and the products of digestion in the rumen, which in turn can affect animal production.

The effects of CT can be measured by comparing treatments with and without the addition of polyethylene glycol (PEG), which binds to and inactivates CT (Jones & Mangan 1977). An experiment with different proportions of minced white clover leaves and flowers incubated *in vitro* with buffered rumen fluid showed that the effects of floral CT on rumen metabolite concentrations were minor relative to the effects of increasing proportions of clover flowers (Chapter 5).

Increasing the percentage of minced clover flowers in the mixture reduced initial soluble protein concentrations; 100% clover flower was less than half that of 100% clover leaf (Figure 5.2). The protein in white clover leaf was rapidly degraded with

ammonia production exceeding microbial utilisation, but when flowers comprised 75% of DM or greater ammonia concentrations may have limited microbial growth. The decreased conversion of plant N to ammonia N with increasing proportions of white clover flower was associated with a lower protein concentration in the DM. Benefits to the cow of reduced ammonia disposal will be offset by the lower nutritive value of clover flowers compared to leaves. However, increasing proportions of white clover flower in a cow's diet is unlikely to affect performance unless white clover is a high proportion of the diet.

Increasing flower proportions did not affect total VFA production, but the proportion of VFA as acetate increased whilst that of propionate and butyrate decreased. This affect was reduced by the floral CT (Chapter 5), so flowers with high CT concentrations will have a higher nutritive value than flowers with low CT concentrations.

The grazing experiments described in Chapter 4 were undertaken with white clover monocultures. In New Zealand, white clover is normally sown in mixed pastures with perennial ryegrass. Chapter 6 evaluated the effects of white clover flowers when mixed with perennial ryegrass leaves on *in vitro* rumen digestion. Increasing the percentage of clover flowers from 0 to 50% decreased the percentage of plant protein appearing as rumen ammonia at 24 hours of incubation from 27 to 13%. White clover flowers had a much greater effect on proteolysis than birdsfoot trefoil (*Lotus corniculatus* L.), with the 50:50 mixture of birdsfoot trefoil and perennial ryegrass having 24% of its plant-N as ammonia-N at 24 hours incubation. These differences may have been due to the high protein concentrations and greater release of DM into solution following mincing of birdsfoot trefoil than of white clover flowers. Therefore, the overall availability of protein for absorption from the intestines may still be greater for mixtures containing birdsfoot trefoil than white clover flowers. Differences between treatments in total VFA concentrations were small, but the lower acetate to propionate ratios from birdsfoot trefoil treatments indicates a more efficient energy capture, a greater availability of lactose precursors and a higher nutritive value than that of white clover flowers (Holmes et al. 2002b,c).

7.5 FURTHER RESEARCH

7.5.1 Effects of grazing management, climate and soil fertility on floral CT concentration

The variation in floral CT concentrations between sampling dates indicate environmental conditions and flower maturity effects are likely to have more control on floral CT concentration than genotype. The effects of these variables (stage of flower development, soil fertility, climate) on white clover floral CT needs to be evaluated to estimate how CT concentrations would vary in different environments. Understanding the effects of harvest interval and soil fertility on sward CT concentrations may enable appropriate management procedures to be devised to maximise CT. Dairy farm profitability relies heavily on maximising pasture production and utilisation (Kidd 2000). Management systems for increasing the CT concentration of white clover would also have to align with these goals.

7.5.2 Survival of white clover cultivars and contamination from buried seed in dairy pastures

Most evaluations of new cultivars run for a maximum of three years, because of high research costs. However, new pastures are expected to persist for longer than this before resowing. Soils from dairy pastures contain high levels of buried white clover seed that can remain viable for 30 years or more (Suckling & Charlton 1978). Plants arising from buried seed may eventually dominate pastures unless new cultivars have superior production and persistence. In a small plot experiment with four white clover cultivars in New Zealand dairy pasture, buried seed contributed 10% of the initial clover population, increasing to 37% one year after sowing (Ledgard et al. 1990). The site had a low level of buried white clover seed (11 kg/ha) compared to that under other New Zealand dairy pastures (3-91 kg/ha; Suckling & Charlton 1978; Ledgard et al. 1988).

The contribution of non-sown white clover to dairy pastures must be evaluated over more than three years to develop a better insight into the performance of new cultivars. New cultivars must be able to compete with plants that germinate from buried seed banks, otherwise some benefits of pasture renewal will be lost and advances in nutritive value will not be captured by grazing animals.

7.5.3 Quantification of the effects of white clover floral CT on milk production

The lower feed quality of white clover flowers relative to clover leaves affected the evaluation of white clover floral CT in the grazing experiments. Although *in vitro* experiments showed increasing clover flower contents reduced the nutritive value of white clover (despite the benefits of CT), cows fed high flowering HT white clover had similar milk production to cows fed Huia white clover. The higher flower content of HT may have masked the potential benefits of a higher CT concentration and milk production differences may have been too small to detect in this experiment. Studies with HT white clover at peak CT concentrations may enable a more accurate determination of the effects of white clover floral CT on milk production. Feeding cows flowering HT white clover and drenching half of the cows with PEG (polyethylene glycol) to inactivate the CT would enable the CT effects to be determined.

7.6 CONCLUSIONS

At currently achievable CT concentrations, white clover flowers are not a suitable source of condensed tannins for increasing animal production or for the prevention of bloat in dairy cows in New Zealand. The poor performance of HT white clover is attributed to the following:

- Higher floral condensed tannin concentrations were not achieved in HT white clover (bred specifically for increased CT concentrations) than in a control white clover cultivar (Grasslands Huia) over 2 years of field monitoring. Floral CT concentrations varied markedly amongst sampling dates, with no clear indication of the cause of variation. A better understanding of this variation is required to be able to manage pastures to optimise CT concentrations.

- Increased flowering in HT white clover compromised its agronomic performance. Herbage production in spring and summer, and stolon growing point densities were lower than for Huia white clover. HT white clover was less competitive and persistent than Huia, leading to the ingress of weeds and volunteer white clover plants. The initial difference in sward CT concentrations

due to increased flowering in HT was lost due to competition from less prolific flowering genotypes.

- Bloat still occurred in cows grazing HT white clover, and low flower densities in spring would exacerbate the problem as that is when bloat is most likely to occur. Bloat was prevented at peak flowering in late spring/early summer, indicating a high flower density will reduce its incidence.

- Herbage CT concentrations in both clovers failed to reach concentrations assumed to increase animal production (20 to 40 g/kg DM). Maximum concentrations were 12 g/kg DM. The CT concentration would be further diluted when white clover is grown in mixed swards with grasses.

- The type of CT (prodelphinidin) present in white clover flowers is considered less beneficial to animal performance than the predominant CT in birdsfoot trefoil (procyanidin).

- *In vitro* effects of CT in white clover flowers (at concentrations of 52 g/kg DM in HT) on rumen digestion were small compared with the effects of increasing flower contents. The increase in acetate production relative to propionate indicates that increasing the proportion of white clover flowers relative to white clover leaf in the diet is unlikely to benefit milk production. The molar percentages of volatile fatty acids produced from incubations of birdsfoot trefoil with perennial ryegrass leaf were more likely to benefit ruminant production than VFA from mixtures of white clover flower with perennial ryegrass leaf.

- The effects of HT clover on rumen metabolite concentrations were small compared to the effects of CT in other legumes. HT white clover failed to show any milk production or compositional benefits over cows grazing Huia white clover. This may be a result of the low dietary CT in HT swards and the lower crude protein concentration, metabolisable energy and digestibility of white clover flowers compared with leaves.

Appendices

APPENDIX 4.2 An example of a grazing schedule, based on the Feb 02 treatment period described in Chapter 4.

Key

HT paddock

Huia paddock

Break fence (each paddock divided into 3 breaks)

Paddock:			
6B	4-Feb **Day 6**	10-Feb **Day 12**	16-Feb **Day 18**
6A	3-Feb **Day 5**	8-Feb **Day 11**	15-Feb **Day 17**
5B	4-Feb **Day 6**	10-Feb **Day 12**	16-Feb **Day 18**
5A	3-Feb **Day 5**	9-Feb **Day 11**	15-Feb **Day 17**
4B	2-Feb **Day 4**	8-Feb **Day 10**	14-Feb **Day 16**
4A	1-Feb **Day 3**	7-Feb **Day 9**	13-Feb **Day 15**
3B	2-Feb **Day 4**	8-Feb **Day 10**	14-Feb **Day 16**
3A	1-Feb **Day 3**	7-Feb **Day 9**	13-Feb **Day 15**

Paddock:	**2B**	**2A**	**1B**	**1A**
	31-Jan **Day 2**	30-Jan **Day 1**	31-Jan **Day 2**	30-Jan **Day 1**
	6-Feb **Day 8**	5-Feb **Day 7**	6-Feb **Day 8**	5-Feb **Day 7**
	12-Feb **Day 14**	11-Feb **Day 13**	12-Feb **Day 14**	11-Feb **Day 13**

APPENDIX 4.3 System of scoring for bloat severity (adapted from Johns 1954).

Score	Description of symptoms
0	No bloat: No distension in left flank.
1	Slight: Distension in left flank, slight pressure.
2	Mild: Marked distension in left paralumbar fossa; well rounded between hip and rib on left side, little or no distension on right side
3	Moderate: Well rounded on left side, drumlike; full on right side; restless
4	Severe: Both sides badly distended; left hip nearly hidden; skin tight; defecation; urination; incoordination; protruding anus; mild respiratory distress.
5	Dead

APPENDIX 4.4 Concentration (mM/L) of volatile fatty acids in the rumen fluid of cows grazing Huia or HT white clover.

Period	Time[1]	Acetate		Propionate		Butyrate		Isobutyrate		Valerate		Isovalerate		Total minor	
		Huia	HT	Huia	HT	Huia	HT	Huia	HT	Huia	HT	Huia	HT	Huia	HT
Dec 01	1	109	110	31.0	30.7	22.1	21.4	2.88	2.92	3.28	3.02	4.80	4.28	10.99	10.20
	2	108	96	32.1	28.9*	21.8	18.6*	2.84	2.64	2.92	2.46	4.50	4.00	10.26	9.09
	3	127	124	40.6	40.6	28.5	27.3	3.86	3.64	4.38	3.92†	6.42	5.60	14.68	13.19
Feb 02	1	83.6	80.8	23.7	21.6	16.2	15.7	3.18	3.20	2.42	2.04	3.72	3.28	9.36	8.51
	2	82.9	82.2	26.2	24.6	17.1	16.3	3.78	3.36	1.68	2.16	4.04	3.62	9.47	9.14
	3	89.7	93.0	31.2	32.4	21.7	22.1	4.16	4.46	3.22	3.24	4.38	3.86	11.78	11.53
Apr 02	1	79.7	76.3	21.4	19.4	13.9	13.4	1.88	1.96	1.96	1.76	3.06	2.76	6.87	6.48
	2	83.9	82.3	20.2	20.5	17.5	17.2	2.36	1.18**	2.20	2.20	3.28	3.02	7.83	6.45*
	3	103.9	94.0	37.1	32.8	27.1	22.6	2.32	3.02	3.60	3.10	4.54	3.54	10.43	9.68
Dec 02	1	65.8	71.8	20.6	22.2	15.5	15.7	1.66	1.74	1.86	2.00	2.50	2.54	6.03	6.28
	2	72.8	70.1	24.2	22.8	17.9	15.9*	1.86	1.70	2.04	2.12	2.72	2.72	6.59	6.53
	3	74.4	90.9**	25.4	32.1**	19.5	23.9*	2.08	2.50**	2.40	3.12*	3.18	3.78*	7.64	9.44**
Apr 03	1	84.1	77.8†	26.2	23.9	15.9	14.3*	2.10	2.20	2.38	2.00*	3.23	2.95	7.73	7.17
	2	78.5	80.6	28.3	27.3	16.8	15.8	2.20	2.30	2.35	2.23	3.13	3.33	7.68	7.88
	3	94.9	93.2	36.6	33.4	24.9	21.2*	3.08	3.05	3.85	3.33	4.83	4.48	11.76	10.88

[1] 1 , 0730 h; 2, 1030 h; 3, 1500 h. See Section 4.3.7 † $P < 0.1$; * $P < 0.05$; ** $P < 0.01$.

APPENDIX 4.5 Covariate adjusted average daily yield (kg/cow/day) of milk protein, fat and lactose of cows grazing Huia or HT white clover for each treatment period.

	Huia	HT	SED	P
Protein yield				
Dec 01	0.70	0.69	0.030	0.679
Feb 02	0.61	0.61	0.025	0.962
Apr 02	0.56	0.59	0.026	0.164
Dec 02	0.89	0.86	0.034	0.316
Apr 03	0.72	0.70	0.026	0.471
Fat yield				
Dec 01	0.85	0.82	0.039	0.608
Feb 02	0.71	0.73	0.021	0.385
Apr 02	0.69	0.72	0.027	0.275
Dec 02	1.01	1.04	0.036	0.405
Apr 03	0.84	0.82	0.035	0.625
Lactose yield				
Dec 01	1.03	1.00	0.042	0.521
Feb 02	0.87	0.89	0.041	0.519
Apr 02	0.74	0.81	0.039	0.093
Dec 02	1.17	1.13	0.029	0.202
Apr 03	0.94	0.89	0.039	0.252

APPENDIX 4.6 Rumen foam volume (mL) in Feb 02, Apr 02 and Dec 02 cow treatment periods reported in Chapter 4.

Period	Date	Cow	Treatment	kg digesta	Digesta DM%	mL foam produced			mL foam/kg digesta		
Time (minutes) since foam collected:						*0*	*30*	*60*	*0*	*30*	*60*
Feb-02	31/1	1765	Huia	3.7	16.5	0	0	0	0	0	0
Feb-02	31/1	2706	Huia	3.6	14.3	40	40	40	11	11	11
Feb-02	31/1	3823	Huia	3.2	14.3	0	0	0	0	0	0
Feb-02	31/1	4328	Huia	3.3	12.1	270	250	240	83	77	74
Feb-02	31/1	6919	Huia	3.2	11.9	370	370	360	117	117	114
Feb-02	31/1	302	HT	3.3	13.2	0	0	0	0	0	0
Feb-02	31/1	727	HT	3.3	14.8	60	45	40	18	14	12
Feb-02	31/1	2801	HT	3.3	13.5	40	40	40	12	12	12
Feb-02	31/1	5775	HT	3.0	12.8	235	235	235	78	78	78
Feb-02	31/1	6921	HT	3.3	15.0	121	120	105	37	37	32
Feb-02	1/2	1765	Huia	3.6	13.6	140	140	140	39	39	39
Feb-02	1/2	2706	Huia	3.5	12.0	180	180	180	52	52	52
Feb-02	1/2	3823	Huia	3.5	14.2	80	60	60	23	17	17
Feb-02	1/2	4328	Huia	3.3	14.4	160	150	150	49	46	46
Feb-02	1/2	6919	Huia	3.3	11.8	530	520	520	163	159	159
Feb-02	1/2	302	HT	3.4	11.4	200	195	195	60	58	58
Feb-02	1/2	727	HT	3.6	14.1	90	80	80	25	22	22
Feb-02	1/2	2801	HT	3.5	11.6	200	200	200	56	56	56
Feb-02	1/2	5775	HT	3.3	12.3	280	280	270	86	86	83
Feb-02	1/2	6921	HT	3.4	14.6	130	120	120	38	35	35
Apr-02	20/3	1765	Huia	3.5	12.8	370	220	210	107	64	61
Apr-02	20/3	3343	Huia	3.5	14.5	340	260	200	99	75	58
Apr-02	20/3	3823	Huia	4.0	15.6	505	300	240	128	76	61
Apr-02	20/3	4328	Huia	3.4	14.2	370	140	120	110	42	36
Apr-02	20/3	6921	Huia	3.2	13.6	140	130	100	44	41	32
Apr-02	20/3	302	HT	3.5	12.6	55	50	50	16	14	14
Apr-02	20/3	2706	HT	3.3	12.7	250	220	220	76	67	67
Apr-02	20/3	2801	HT	3.0	14.4	205	100	100	68	33	33
Apr-02	20/3	5272	HT	3.0	15.5	230	150	120	78	51	47
Apr-02	20/3	6919	HT	3.3	14.5	170	110	100	52	34	31
Apr-02	21/3	1765	Huia	3.4	13.6	80	80	70	24	24	21
Apr-02	21/3	3343	Huia	3.6	13.9	150	120	110	42	39	31
Apr-02	21/3	3823	Huia	3.6	13.0	180	120	100	50	33	28
Apr-02	21/3	4328	Huia	3.4	13.9	200	180	160	60	54	48
Apr-02	21/3	6921	Huia	3.0	13.0	160	120	110	54	41	37
Apr-02	21/3	302	HT	3.2	12.7	100	90	60	32	29	19
Apr-02	21/3	2706	HT	3.1	13.8	290	280	270	94	90	87
Apr-02	21/3	2801	HT	3.3	13.1	160	160	130	48	48	39
Apr-02	21/3	5272	HT	3.3	12.6	220	190	180	68	58	55
Apr-02	21/3	6919	HT	3.7	12.0	380	360	330	102	97	89
Apr-02	22/3	1765	Huia	3.2	14.5	100	90	90	32	29	29
Apr-02	22/3	3343	Huia	3.2	13.0	160	130	110	51	41	35
Apr-02	22/3	3823	Huia	3.8	14.9	40	30	30	11	8	8
Apr-02	22/3	4328	Huia	3.5	13.6	75	50	50	22	15	15
Apr-02	22/3	6921	Huia	3.2	13.0	200	190	180	63	60	57
Apr-02	22/3	302	HT	3.0	14.2	160	150	150	53	50	50
Apr-02	22/3	2706	HT	3.3	11.5	200	190	190	62	58	58
Apr-02	22/3	2801	HT	3.1	14.0	260	250	230	84	80	74
Apr-02	22/3	5272	HT	3.2	13.3	100	100	70	31	31	22
Apr-02	22/3	6919	HT	3.2	15.4	280	250	240	87	78	75

Period	Date	Cow	Treatment	kg digesta	DM%	mL foam produced			mL foam/kg digesta		
Time (minutes) since foam collected:						*0*	*30*	*60*	*0*	*30*	*60*
Dec-02	19/11	302	Huia	3.3	12.4	25	25	10	7.7	7.7	3.1
Dec-02	19/11	5272	Huia	3.3	12.0	1	0	0	0.3	0.0	0.0
Dec-02	19/11	7915	Huia	3.2	12.4	40	10	5	12.6	3.2	1.6
Dec-02	19/11	7920	Huia	3.7	12.7	60	40	20	16.3	10.9	5.4
Dec-02	19/11	8223	Huia	3.0	13.5	60	40	20	20.2	13.5	6.7
Dec-02	19/11	2706	HT	3.2	11.8	50	40	40	15.5	12.4	12.4
Dec-02	19/11	2801	HT	3.7	11.0	50	40	30	13.4	10.7	8.1
Dec-02	19/11	6919	HT	2.9	11.7	10	5	5	3.5	1.7	1.7
Dec-02	19/11	7926	HT	3.0	13.2	40	20	20	13.5	6.7	6.7
Dec-02	19/11	8630	HT	3.0	12.7	30	20	20	9.9	6.6	6.6
Dec-02	20/11	302	Huia	3.9	12.3	30	25	25	7.8	6.5	6.5
Dec-02	20/11	5272	Huia	3.9	11.8	80	70	40	20.7	18.1	10.4
Dec-02	20/11	7915	Huia	3.6	14.1	70	50	40	19.6	14.0	11.2
Dec-02	20/11	7920	Huia	2.9	12.6	20	10	10	6.8	3.4	3.4
Dec-02	20/11	8223	Huia	3.7	11.7	220	220	200	59.9	59.9	54.5
Dec-02	20/11	2706	HT	3.8	12.7	180	140	110	47.7	37.1	29.2
Dec-02	20/11	2801	HT	2.9	11.8	140	100	70	47.9	34.2	23.9
Dec-02	20/11	6919	HT	3.3	11.3	1	1	0	0.3	0.3	0.0
Dec-02	20/11	7926	HT	3.9	12.0	50	40	30	12.9	10.3	7.8
Dec-02	20/11	8630	HT	3.1	11.4	40	20	20	13.0	6.5	6.5
Dec-02	21/11	302	Huia	3.3	11.9	70	60	50	21.1	18.1	15.1
Dec-02	21/11	5272	Huia	3.7	11.6	120	110	100	32.8	30.0	27.3
Dec-02	21/11	7915	Huia	4.5	12.5	10	5	5	2.2	1.1	1.1
Dec-02	21/11	7920	Huia	2.9	11.2	110	95	85	37.7	32.5	29.1
Dec-02	21/11	8223	Huia	3.3	11.3	40	30	30	12.0	9.0	9.0
Dec-02	21/11	2706	HT	3.3	12.2	120	100	90	36.1	30.1	27.1
Dec-02	21/11	2801	HT	3.2	11.2	120	110	90	37.2	34.1	27.9
Dec-02	21/11	6919	HT	3.2	11.2	20	15	15	6.3	4.7	4.7
Dec-02	21/11	7926	HT	3.1	12.5	20	15	5	6.4	4.8	1.6
Dec-02	21/11	8630	HT	3.2	12.9	50	45	40	15.8	14.2	12.6

APPENDIX 5 Preparation of McDougall's buffer (McDougall 1958) and reducing agent, and collection and use of rumen fluid for *in vitro* incubations.

McDougall's buffer (artificial saliva)

McDougall's buffer is added to the incubation bottles to help maintain a constant pH. The buffer was prepared the day before incubations, and was made in a 2 L batch for treatments without PEG, and a 1 L batch for the treatments with PEG. The buffer was placed in hot water and warmed to approximately 39°C, before gassing with carbon dioxide for approximately 45 minutes and dispensing into sample bottles. The following ingredients were used for each litre of buffer and the volume made up to l L with water:

- 9.8 g $NaHCO_3$
- 3.67 g Na_2HPO_4 (anhydrous)
- 0.47 g NaCl
- 0.57 g KCl
- 0.125g $MgCl_2.6H_20$

McDougall's buffer with PEG

For treatments requiring PEG, the preceding recipe was used with 2 g of PEG 4000 (molecular weight 3500 daltons) (Union Carbide Co.) added. The amount of PEG required was calculated as follows:

- The weight of PEG added should be approximately twice the weight of the CT in the sample (Barry & Forss 1983).
- White clover flowers are approximately 5% CT, flowers make up half of the treatment with PEG, therefore 2.5 % of the treatment with PEG is CT.
- Weight of sample in bottle = 0.5 g DM.
- Weight of CT in bottle = 0.5 g x 2.5 % = 0.0125 g CT.
- Therefore, there is 0.0125 g CT mixed with 12 mL (0.012 L) of buffer.
- PEG required per L of buffer = (0.0125÷0.012) x 2 = 2 g PEG/L buffer.

The 50R+50LC+PEG treatment described in Chapter 6 was estimated to have a similar CT concentration, and so the same PEG buffer was used.

Reducing agent (cysteine sulphide)

Reducing agent is added to the incubations to remove residual oxygen and maintain an anaerobic environment. The reducing agent was prepared immediately before dispensing by mixing the following:

- 315 mg cysteine hydrochloride
- 48 mL water
- 2 mL 1N NaOH
- 315 mg sodium sulphide ($Na_2S.9H_20$) – crystals were rinsed in water and blotted to achieve the appropriate dry weight.

Collection and use of rumen fluid

Rumen inoculum was taken from three rumen-fistulated lactating Friesian diary cows fed an unrestricted diet of mixed perennial ryegrass/white clover, to minimise variation attributed to differences between cows in microbial populations (Weimer et al. 1999). Samples were taken approximately 2 hours after the start of morning grazing as follows.

Approximately 2 kg of rumen digesta was removed from each cow and the fluid squeezed into a single bucket through cheese cloth and transferred into a preheated vacuum flask. The flask was filled to minimise oxygen in the head space, and was immediately used for inoculation of *in vitro* incubations.

Two people were required for addition of rumen fluid to the incubation bottles to ensure a rapid inoculation. One person removed and replaced the screw caps, while the other person pipetted 3 mL aliquots of rumen fluid.

NB: No detergent was used when cleaning any glassware used in the experiments to avoid killing rumen bacteria during incubations.

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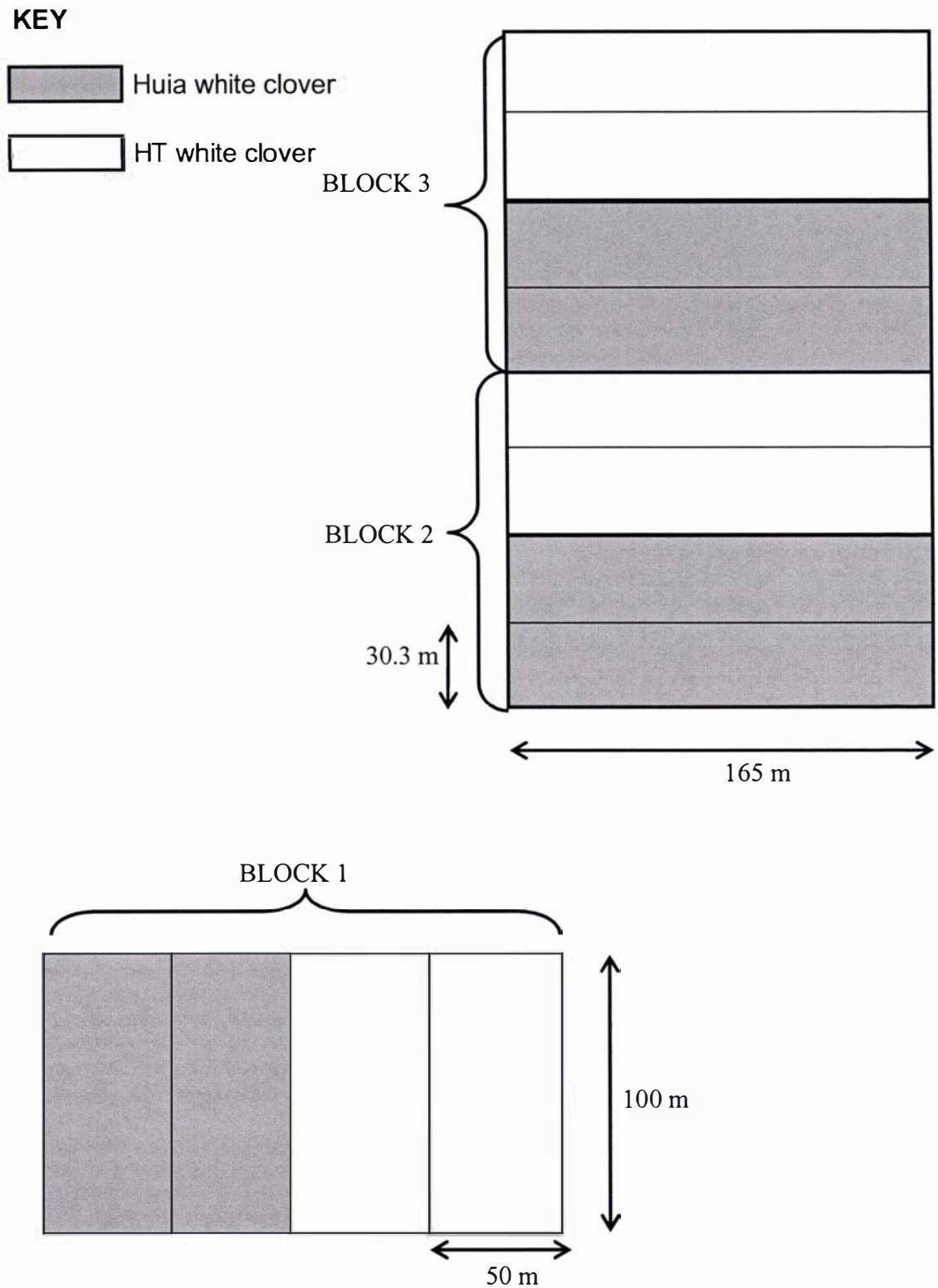
APPENDIX 3.2 Experimental layout of paddocks used in experiments that are presented in Chapters 3 and 4.



APPENDIX 3.3 Calibration and validation statistics for near-infrared reflectance spectroscopy determination of free, protein bound and fibre bound condensed tannin (CT) concentrations in white clover flower heads.

Constituent	N	Mean	SEC	RSQ	SECV	1-VR
Free CT	30	3.36	0.14	0.99	0.23	0.98
Protein bound CT	37	1.90	0.19	0.92	0.34	0.74
Fibre bound CT	33	0.27	0.03	0.96	0.05	0.84

Where N = Number of samples
SEC = Standard Error of Calibration (e.g. free CT 3.36 ± 0.14%)
RSQ = R Squared
SECV = Standard Error of Cross Validation (e.g. free CT 3.36 ± 0.23%)
1-VR = Cross Validation RSQ

APPENDIX 3.4 Method for micro-cyanogenesis testing of white clover. Adapted from Corkill (1940).

Equipment

1.5 mL safelock microcentrifuge tubes
microcentrifuge tube racks
Whatman No. 1 filter paper
Freshly prepared sodium picrate
Micropipette and tips
Tweezers
Toluene
Fume hood and safety glasses
Freshly picked clover leaves

Preparation of sodium picrate

Weigh 5 g of sodium carbonate into approximately 50 mL water.
Weigh 0.5 g picric acid onto a plastic dish. Take care not to spill acid or let it dry out.
Rinse picric acid from dish into sodium carbonate solution with water, making volume up to 100 mL, place lid on container, swirl to mix.

Sample preparation and analysis

For each leaf to be tested, place one microcentrifuge tube into a microcentrifuge rack.
Cut filter paper into small squares (approximately 8 mm by 8 mm) then insert one filter paper square into the lid of each microcentrifuge tube.
Remove one leaflet from each clover leaf. Place leaflet into microcentrifuge tube, and push to the bottom of the tube with tweezers.
Transfer samples and toluene and picric acid to a fume hood.
Add 10μL of picric acid to the filter paper, this will turn the filter paper yellow.
Add 10μL of toluene to the leaflet, to enhance enzyme activity.
Close lid of microcentrifuge tubes and leave overnight for reaction to occur.
The next morning, count the number of tubes that have had a cyanogenic reaction. This is indicated by the filter paper changing from yellow to various degrees of red.

APPENDIX 3.5 Monthly rainfall, average grass minimum temperature and maximum air temperature from sowing of white clover (April 2001) to the end of agronomic measurements (November 2003) (Chapter 3). Ten-year averages are presented in parentheses. Data were recorded at the Ruakura Climatological Station, located 4 km from the experimental site.

Month	Monthly rainfall (mm)	Monthly mean grass minimum (°C)	Monthly mean maximum air temperature (°C)
2001			
April	71 (101)	5.7 (5.5)	20.0 (19.9)
May	137 (95)	6.6 (3.5)	17.6 (16.9)
June	40 (116)	0.2 (1.2)	14.5 (14.2)
July	90 (143)	-0.8 (0.8)	13.4 (13.8)
August	56 (128)	2.1 (1.3)	15.2 (14.4)
September	38 (100)	3.1 (2.5)	17.0 (16.0)
October	66 (85)	7.2 (5.1)	19.9 (18.0)
November	134 (96)	8.5 (6.5)	20.4 (19.6)
December	21 (75)	12.8 (8.2)	22.3 (21.7)
2002			
January	94 (66)	10.0 (9.4)	23.8 (23.9)
February	22 (65)	9.2 (10.1)	23.7 (24.8)
March	96 (90)	9.0 (7.6)	23.4 (22.6)
April	46	5.5	20.3
May	83	5.2	17.6
June	119	6.5	16.3
July	112	2.4	14.9
August	87	2.1	15.4
September	75	4.9	16.6
October	71	2.4	17.3
November	79	6.2	18.6
December	108	9.4	21.9
2003			
January 2003	103	9.4	23.5
February 2003	44	9.1	23.9
March 2003	123	9.9	23.2
April 2003	46	6.0	20.8
May 2003	88	4.2	18.1
June 2003	146	4.1	16.1
July 2003	71	-0.5	13.6
August 2003	50	1.5	15.4
September 2003	147	5.5	16.9
October 2003	85	3.3	17.8
November 2003	121	5.6	18.9